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**BERESKIN & PARR**

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**Title: PREPARATION OF HETEROLOGOUS PROTEINS ON OIL BODIES**

**Inventors: Maurice M. Moloney and Gijs van Rooijen**

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5 **CROSS-REFERENCE TO RELATED APPLICATIONS**

The present application is a continuation-in-part of USSN 09/210,843 that was filed December 15, 1998, which is a continuation-in-part of USSN 08/846,021 that was filed April 25, 1997 (now U.S. Patent No. 5,948,682), which is a continuation-in-part of USSN 08/366,783 that was filed on December 10 30, 1994 (now U.S. Patent No. 5,650,554), which is a continuation-in-part of USSN 08/142,418 that was filed November 16, 1993 (now abandoned), which is a continuation-in-part of USSN 07/659,835 that was filed on February 22, 1991 (now abandoned), all of which are incorporated by reference in their entirety.

**FIELD OF THE INVENTION**

15 The present invention relates to novel methods for the preparation of heterologous proteins.

**BACKGROUND OF THE INVENTION**

Many very diverse methods have been tested for the production of recombinant molecules of interest and commercial value. Different 20 organisms that have been considered as hosts for foreign protein expression include single celled organisms such as bacteria and yeasts, cells and cell cultures of animals, fungi and plants and whole organisms such as plants, insects and transgenic animals.

The use of fermentation techniques for large scale production of 25 bacteria, yeasts and higher organism cell cultures is well established. The capital costs associated with establishment of the facility and the costs of maintenance are negative economic factors. Although the expression levels of proteins that can be achieved are high, energy inputs and protein purification costs can greatly increase the cost of recombinant protein production.

30 The production of a variety of proteins of therapeutic interest has been described in transgenic animals, however the cost of establishing substantial manufacturing is prohibitive for all but high value proteins. Numerous foreign proteins have been expressed in whole plants and selected

plant organs. Methods of stably inserting recombinant DNA into plants have become routine and the number of species that are now accessible to these methods has increased greatly.

Plants represent a highly effective and economical means to produce recombinant proteins as they can be grown on a large scale with modest cost inputs and most commercially important species can now be transformed. Although the expression of foreign proteins has been clearly demonstrated, the development of systems with commercially viable levels of expression coupled with cost effective separation techniques has been limited.

The production of recombinant proteins and peptides in plants has been investigated using a variety of approaches including transcriptional fusions using a strong constitutive plant promoter (e.g., from cauliflower mosaic virus (Sijmons et al., 1990, Bio/Technology, 8:217-221); transcriptional fusions with organ specific promoter sequences (Radke et al., 1988, Theoret. Appl. Genet., 75:685-694); and translational fusions which require subsequent cleavage of a recombinant protein (Vanderkerckove et al., 1989, Bio/Technology, 7:929-932).

Foreign proteins that have been successfully expressed in plant cells include proteins from bacteria (Fraley et al., 1983, Proc. Natl. Acad. Sci. USA, 80:4803-4807), animals (Misra and Gedamu, 1989, Theor. Appl. Genet., 78:161-168), fungi and other plant species (Fraley et al., 1983, Proc. Natl. Acad. Sci. USA, 80:4803-4807). Some proteins, predominantly markers of DNA integration, have been expressed in specific cells and tissues including seeds (Sen Gupta-Gopalan et al., 1985, Proc. Natl. Acad. Sci. USA, 82:3320-3324); Radke et al., 1988, Theor. Appl. Genet., 75:685-694). Seed specific research has been focused on the use of seed-storage protein promoters as a means of deriving seed-specific expression. Using such a system, Vanderkerckove et al., (1989, Bio/Technol., 7:929-932) expressed the peptide leu-enkephalin in seeds of *Arabidopsis thaliana* and *Brassica napus*. The level of expression of this peptide was quite low and it appeared that expression of this peptide was limited to endosperm tissue.

It has been generally shown that the construction of chimeric genes which contain the promoter from a given regulated gene and a coding sequence of a reporter protein not normally associated with that promoter gives

rise to regulated expression of the reporter. The use of promoters from seed-specific genes for the expression of recombinant sequences in seed that are not normally expressed in a seed-specific manner have been described.

5 Sengupta-Gopalan et al., (1985, Proc. Natl. Acad. Sci. USA, 82:3320-3324) reported expression of a major storage protein of french bean, called  $\beta$ -phaseolin, in tobacco plants. The gene expressed correctly in the seeds and only at very low levels elsewhere in the plant. However, the constructs used by Sengupta-Gopalan were not chimeric. The entire  $\beta$ -phaseolin gene including the native 5'-flanking sequences were used. Subsequent experiments with other  
10 species (Radke et al., 1988, Theor. App. Genet. 75:685-694) or other genes (Perez-Grau, L., Goldberg, R.B., 1989, Plant Cell, 1:1095-1109) showed the fidelity of expression in a seed-specific manner in both *Arabidopsis* and *Brassica*. Radke et al., (1988, *vide supra*), used a "tagged" gene i.e., one containing the entire napin gene plus a non-translated "tag".

15 The role of the storage proteins is to serve as a reserve of nitrogen during seed germination and growth. Although storage protein genes can be expressed at high levels, they represent a class of protein whose complete three-dimensional structure appears important for proper packaging and storage. The storage proteins generally assemble into multimeric units which are  
20 arranged in specific bodies in endosperm tissue. Perturbation of the structure by the addition of foreign peptide sequences leads to storage proteins unable to be packaged properly in the seed.

In addition to nitrogen, the seed also stores lipids. The storage of lipids occurs in oil or lipid bodies. Analysis of the contents of lipid bodies has  
25 demonstrated that in addition to triglyceride and membrane lipids, there are also several polypeptides/proteins associated with the surface or lumen of the oil body (Bowman-Vance and Huang, 1987, J. Biol. Chem., 262:11275-11279, Murphy et al., 1989, Biochem. J., 258:285-293, Taylor et al., 1990, Planta, 181:18-26). Oil-body proteins have been identified in a wide range of taxonomically  
30 diverse species (Moreau et al., 1980, Plant Physiol., 65:1176-1180; Qu et al., 1986, Biochem. J., 235:57-65) and have been shown to be uniquely localized in oil-bodies and not found in organelles of vegetative tissues. In *Brassica napus*

(rapeseed, canola) there are at least three polypeptides associated with the oil-bodies of developing seeds (Taylor et al., 1990, *Planta*, 181:18-26).

The oil bodies that are produced in seeds are of a similar size (Huang A.H.C., 1985, in *Modern Meths. Plant Analysis*, Vol. 1:145-151 Springer-Verlag, Berlin). Electron microscopic observations have shown that the oil-bodies are surrounded by a membrane and are not freely suspended in the cytoplasm. These oil-bodies have been variously named by electron microscopists as oleosomes, lipid bodies and spherosomes (Gurr MI., 1980, in *The Biochemistry of Plants*, 4:205-248, Acad. Press, Orlando, Fla). The oil-bodies of the species that have been studied are encapsulated by an unusual "half-unit" membrane comprising, not a classical lipid bilayer, but rather a single amphophilic layer with hydrophobic groups on the inside and hydrophilic groups on the outside (Huang A.H.C., 1985, in *Modern Meths. Plant Analysis*, Vol. 1:145-151 Springer-Verlag, Berlin).

The numbers and sizes of oil-body associated proteins may vary from species to species. In corn, for example, there are two immunologically distinct polypeptide classes found in oil-bodies (Bowman-Vance and Huang, 1988, *J. Biol. Chem.*, 263:1476-1481). Oleosins have been shown to comprise alternate hydrophilic and hydrophobic regions (Bowman-Vance and Huang, 1987, *J. Biol. Chem.*, 262:11275-11279). The amino acid sequences of oleosins from corn, rapeseed, and carrot have been obtained. See Qu and Huang, 1990, *J. Biol. Chem.*, 265:2238-2243, Hatzopoulos et al., 1990, *Plant Cell*, 2:457-467, respectively. In an oilseed such as rapeseed, oleosin may comprise between 8% (Taylor et al., 1990, *Planta*, 181:18-26) and 20% (Murphy et al., 1989, *Biochem.J.*, 258:285-293) of total seed protein. Such a level is comparable to that found for many seed storage proteins.

Genomic clones encoding oil-body proteins with their associated upstream regions have been reported for several species, including maize (*Zea mays*, Bowman-Vance and Huang, 1987, *J. Biol. Chem.*, 262:11275-11279; and Qu Huang, 1990, *J. Biol. Chem.*, 265:2238-2243) and carrot (Hatzopoulos et al., 1990, *Plant Cell*, 2:457-467). cDNAs and genomic clones have also been reported for cultivated oilseeds, *Brassica napus* (Murphy, et al., 1991, *Biochem. Biophys. Acta*,

1088:86-94; and Lee and Huang, 1991, Plant Physiol 96:1395-1397), sunflower (Cummins and Murphy, 1992, Plant Molec. Biol. 19:873-878) soybean ( Kalinski et al., 1991, Plant Molec. Biol. 17: 1095-1098), and cotton (Hughes et al., 1993, Plant Physiol 101:697-698). Reports on the expression of these oil-body protein genes in developing seeds have varied. In the case of *Zea mays*, transcription of genes encoding oil-body protein isoforms began quite early in seed development and were easily detected 18 days after pollination. In non-endospermic seeds such as the dicotyledonous plant *Brassica napus* (canola, rapeseed), expression of oil-body protein genes seems to occur later in seed development (Murphy, et al., 1989, Biochem. J., 258:285-293) compared to corn.

A maize oleosin has been expressed in seed oil bodies in *Brassica napus* transformed with a *Zea mays* oleosin gene. The gene was expressed under the control of regulatory elements from a *Brassica* gene encoding napin, a major seed storage protein. The temporal regulation and tissue specificity of expression was reported to be correct for a napin gene promoter/terminator (Lee et al., 1991, Proc. Natl. Acad. Sci. USA, 88:6181-6185).

Thus the above demonstrates that oil body proteins (or oleosins) from various plant sources share a number of similarities in both structure and expression. However, at the time of the above references it was generally believed that modifications to oleosins or oil body proteins would likely lead to aberrant targeting and instability of the protein product. (Vande Kerckhove et al., 1989. Bio/Technology, 7:929-932; Radke et al., 1988. Theor. and Applied Genetics, 75:685-694; and Hoffman et al., 1988. Plant Mol. Biol. 11:717-729).

#### SUMMARY OF THE INVENTION

The present invention describes the use of an oil body protein gene to target the expression of a heterologous polypeptide, to an oil body in a host cell. The unique features of both the oil body protein and the expression patterns are used in this invention to provide a means of synthesizing commercially important proteins on a scale that is difficult if not impossible to achieve using conventional systems of protein production.

In particular, the present invention provides a method for the expression of a heterologous polypeptide by a host cell said method comprising:

a) introducing into a host cell a chimeric nucleic acid sequence comprising: 1) a first nucleic acid sequence capable of regulating the transcription in said host cell of 2) a second nucleic acid sequence, wherein said second sequence encodes a fusion polypeptide and comprises (i) a nucleic acid sequence encoding a sufficient portion of an oil body protein gene to provide targeting of the fusion polypeptide to a lipid phase linked in reading frame to (ii) a nucleic acid sequence encoding the heterologous polypeptide; and 3) a third nucleic acid sequence encoding a termination region functional in the host cell; and b) growing said host cell to produce the fusion polypeptide.

10           The present invention also provides a method for the production and release of a heterologous polypeptide from a fusion polypeptide associated with a plant oil body fraction during seed germination and plant seedling growth, said method comprising: a) introducing into a plant cell a first chimeric nucleic acid sequence comprising: 1) a first nucleic acid sequence capable of  
15   regulating the transcription in said plant cell of 2) a second nucleic acid sequence wherein said nucleic acid second sequence encodes a fusion polypeptide and comprises (i) a nucleic acid sequence encoding a sufficient portion of an oil body protein gene to provide targeting of the fusion polypeptide to an oil body, linked in reading frame to (ii) a nucleic acid sequence encoding the heterologous  
20   polypeptide and (iii) a linker nucleic acid sequence encoding an amino acid sequence that is specifically cleavable by enzymatic means wherein said linker nucleic acid sequence (iii) is located between said nucleic acid sequence (i) encoding the oil body protein and said nucleic acid sequence (ii) encoding the heterologous polypeptide; and 3) a third nucleic acid sequence encoding a  
25   termination region; b) sequentially or concomitantly introducing into the genome of said plant a second chimeric nucleic acid sequence comprising: 1) a first nucleic acid sequence capable of regulating the transcription specifically during seed germination and seed growth of 2) a second nucleic acid sequence encoding a specific enzyme that is capable of cleaving the linker nucleic acid  
30   sequence of said first chimeric nucleic acid sequence; and 3) a third nucleic acid sequence encoding a termination region; c) regenerating a plant from said plant cell and growing said plant to produce seed whereby said fusion polypeptide is

expressed and associated with oil bodies and d) allowing said seed to germinate wherein said enzyme in said second chimeric nucleic acid sequence is expressed and cleaves the heterologous polypeptide from the fusion polypeptide associated with the oil bodies during seed germination and early seedling growth.

The present invention further provides a method for producing an altered seed meal by producing a heterologous polypeptide in association with a plant seed oil body fraction, said method comprising: a) introducing into a plant cell a chimeric nucleic acid sequence comprising: 1) a first nucleic acid sequence capable of regulating the transcription in said plant cell of 2) a second nucleic acid sequence wherein said second sequence encodes a fusion polypeptide and comprises (i) a nucleic acid sequence encoding a sufficient portion of an oil body protein gene to provide targeting of the fusion polypeptide to an oil body, linked in reading frame to (ii) a nucleic acid sequence encoding the heterologous polypeptide and 3) a third nucleic acid sequence encoding a termination region; b) regenerating a plant from said plant cell and growing said plant to produce seed whereby said heterologous polypeptide is expressed and associated with oil bodies; and c) crushing said seed and preparing an altered seed meal.

The present invention yet also provides a method of preparing an enzyme in a host cell in association with an oil body and releasing said enzyme from the oil body, said method comprising: a) transforming a host cell with a chimeric nucleic acid sequence comprising: 1) a first nucleic acid sequence capable of regulating the transcription of 2) a second nucleic acid sequence, wherein said second sequence encodes a fusion polypeptide and comprises (i) a nucleic acid sequence encoding a sufficient portion of an oil body protein gene to provide targeting of the fusion polypeptide to an oil body; (ii) a nucleic acid sequence encoding an enzyme and (iii) a linker nucleic acid sequence located between said nucleic acid sequence (i) encoding the oil body and said nucleic acid sequence (ii) encoding the enzyme and encoding an amino acid sequence that is cleavable by the enzyme encoded by the nucleic acid sequence (ii); and 3) a third nucleic acid sequence encoding a termination region functional in said host cell b) growing the host cell to produce the fusion polypeptide under conditions

such that enzyme is not active; c) recovering the oil bodies containing the fusion polypeptide; and d) altering the environment of the oil bodies such that the enzyme is activated and cleaves itself from the fusion polypeptide.

The present invention further provides a method for the  
5 expression of a heterologous polypeptide by a host cell in association with an oil body and separating said heterologous polypeptide from the oil body, said method comprising: a) transforming a first host cell with a first chimeric nucleic acid sequence comprising: 1) a first nucleic acid sequence capable of regulating the transcription in said host cell of 2) a second nucleic acid sequence, wherein  
10 said second sequence encodes a first fusion polypeptide and comprises (i) a nucleic acid sequence encoding a sufficient portion of an oil body protein gene to provide targeting of the first fusion polypeptide to a lipid phase linked in reading frame to (ii) a nucleic acid sequence encoding the heterologous polypeptide; and (iii) a linker nucleic acid sequence encoding an amino acid  
15 sequence that is specifically cleavable by enzymatic means wherein said linker nucleic acid sequence (iii) is located between said (i) nucleic acid sequence encoding the oil body protein and said (ii) nucleic acid sequence encoding the heterologous polypeptide; and 3) a third nucleic acid sequence encoding a termination region functional in the host cell; and b) transforming a second host  
20 cell with a second chimeric nucleic acid sequence comprising: 1) a first nucleic acid sequence capable of regulating the transcription specifically during seed germination and seed growth of 2) a second nucleic acid sequence wherein said second sequence encodes a second fusion polypeptide and comprises (i) a nucleic acid sequence encoding a sufficient portion of an oil body protein gene to  
25 provide targeting of the second fusion polypeptide to a lipid phase linked in reading frame to a nucleic acid sequence, encoding a specific enzyme that is capable of cleaving the linker nucleic acid sequence of said first chimeric nucleic acid sequence; and 3) a third nucleic acid sequence encoding a termination region; c) growing said first host cell under conditions such that the first fusion  
30 polypeptide is expressed and associated with the oil bodies to produce a first oil body fraction containing the first recombinant fusion polypeptide; d) growing said second host cell under conditions such that the second fusion polypeptide is

expressed and associated with the oil bodies to product a second oil body fraction containing the second recombinant fusion polypeptide; e) contacting the first oil body fraction of step (c) with the second oil body fraction of step (d) under conditions such that the enzyme portion of the second fusion polypeptide  
5 cleaves the heterologous polypeptide from the first fusion polypeptide.

The present invention also provides a chimeric nucleic acid sequence encoding a fusion polypeptide, capable of being expressed in association with an oil body of a host cell comprising: 1) a first nucleic acid sequence capable of regulating the transcription in said host cell of 2) a second  
10 nucleic acid sequence, wherein said second sequence encodes a fusion polypeptide and comprises (i) a nucleic acid sequence encoding a sufficient portion of an oil body protein gene to provide targeting of the fusion polypeptide to a lipid phase linked in reading frame to (ii) a nucleic acid sequence encoding a heterologous polypeptide; and 3) a third nucleic acid  
15 sequence encoding a termination region functional in the host cell.

The present invention also includes a fusion polypeptides encoded for by a chimeric nucleic acid sequence comprising (i) a nucleic acid sequence encoding a sufficient portion of an oil body protein to provide targeting of the fusion polypeptide to an oil body linked in reading frame to (ii) a nucleic acid  
20 sequence encoding a heterologous polypeptide.

The invention further provides methods for the separation of heterologous proteins from host cell components by partitioning of the oil body fraction and subsequent release of the heterologous protein via specific cleavage of the heterologous protein - oil body protein fusion. Optionally a cleavage site  
25 may be located prior to the N-terminus and after the C-terminus of the heterologous polypeptide allowing the fusion polypeptide to be cleaved and separated by phase separation into its component peptides. This production system finds utility in the production of many proteins and peptides such as those with pharmaceutical, enzymic, rheological and adhesive properties.

30 The processing of a wide variety of materials using enzymes has enormous commercial potential. The present invention provides for methods to produce recombinant enzymes in mass quantities which can be separated from

cellular components by partitioning of the oil-body fraction. The enzyme of interest may be cleaved from the oil body protein or may be used in association with the oil-body fraction. Enzymes fused to an oil body protein in an oil-body fraction represent a type of immobilized and reusable enzyme system.

5 Immobilized enzyme systems have been developed in association with various inert support matrices for many industrial purposes including cellulose beads, plastic matrixes and other types of inert materials. Enzymes attached to oil-bodies can be mixed with solutions containing enzyme substrates and subsequently recovered by floatation and partitioning of the oil-body fraction  
10 and reused.

In addition to the production and isolation of recombinant proteins from plants, the present invention also contemplates methods for crop improvement and protection. The nutritional quality of seeds has been improved by the addition of proteins with high levels of essential amino acids  
15 (DeClercq et al., 1990, Plant Physiol. 94:970-979) and enzymes such as lauroyl-ACP thioesterase from *Umbellularia californica* that affect lipid composition (US Patent 5,298,421). To date these seed modifications have only been conducted using seed storage gene promoters that may have inherent limitations. Use of  
20 oil body protein regulatory sequences provides an additional means by which to accomplish such modifications.

Insect predation and fungal diseases of crop plants represent two of the largest causes of yield losses. A number of strategies dependent on transformation and expression of recombinant proteins in plants have been advanced for the protection of plants from insects and fungi (Lamb et al., 1992,  
25 Bio/Technology 11:1436-1445). These strategies are exemplified by the expression of peptide inhibitors of insect digestive enzymes such as cowpea trypsin inhibitor (Hoffman et al., 1992, J. Economic Entomol. 85: 2516-1522) bacterial or arachnid protein toxins (Gordon and Zlotkin, 1993, FEBS Lett., 315:125-128) and the expression of chitinase enzymes for the digestion of fungal  
30 cell walls (Broglie et al., 1991, Science 254: 5035, 1194-1197; Benhamou et al., 1993, Plant Journal 2:295-305; Dunsmuir et al., 1993, In Advances in molecular genetics of plant-microbe interactions, Vol2. pp 567-571, Nester, E.W. and Verma, D.P.S.

eds.). The use of oil body proteins to localize specific polypeptides that afford crop protection allows one to develop novel strategies to protect vulnerable germinating seeds.

The use of oil body whose expression is limited to pollen allows one to alter the function of pollen to specifically control male fertility. One may use promoter sequences from such oil body to specifically express recombinant proteins that will alter the function of pollen. One such example is the use of such promoters to control the expression of novel recognition proteins such as the self-incompatibility proteins. Additional uses are contemplated including expression of oil body fusion proteins in pollen that are toxic to pollen. Seed specific oil body may be used to alter female fertility.

The methods described above are not limited to heterologous proteins produced in plant seeds as oil body proteins may also be found in association with oil bodies in other cells and tissues. Additionally the methods are not limited to the recovery of heterologous proteins produced in plants because the extraction and release methods can be adapted to accommodate oil body protein-heterologous protein fusions produced in any cell type or organism. An extract containing the fusion protein is mixed with additional oleosins and appropriate tri-glycerides and physical conditions are manipulated to reconstitute the oil-bodies. The reconstituted oil-bodies are separated by floatation and the recombinant proteins released by the cleavage of the junction with oleosin.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows a schematic representation of the types of oil body protein fusions that are contemplated as methods of the invention for the fusion of oil-body protein genes with genes encoding foreign polypeptides. IA is a C-terminal fusion of a desired polypeptide to a oil body protein; IB is an N-terminal fusion of a desired polypeptide to oil body protein; IC is an internal fusion of a desired polypeptide within oil body protein; and ID is an inter-dimer translational fusion of desired polypeptide enclosed between two substantially complete oil body protein targeting sequences. Each fusion is shown in a linear diagrammatic form and in the configuration predicted when specifically

associated with the oil body. In both the linear and oil body associated form, the oil body coding sequence that specifically targets the protein to the oil body is shown as a single thin line, a solid circle represents a protease recognition motif; a corkscrew line represents a native C- or N-terminal of a oil body protein and a  
5 inserted coding region is represented by an open box. The oil body is represented as a simple circle.

Figure 2 shows the nucleotide sequence (SEQ ID NO.1) and deduced amino acid sequence (SEQ ID NO.2 and NO. 3) of an oil-body protein gene that codes for a 18 KDa oleosin from *Arabidopsis thaliana*. The intron  
10 sequence is printed in lower case. The predicted amino acid sequence is shown in single letter code.

Figure 3 shows a schematic representation of the construction of pOleoP1.

Figure 4 shows the nucleotide sequence (SEQ ID NO.4) of a *B. napus* oleosin cDNA clone and the predicted amino acid sequence (SEQ ID  
15 NO.5).

Figure 5 describes the construction of a oleosin/GUS fusion for expression in *E.coli*.

Figure 6 shows the nucleotide sequence (SEQ.ID.NO.6) and deduced amino acid (SEQ.ID.NO.7 and NO.8) sequence of the 2.7 kbp *HindIII* fragment of pSBSOTPTNT containing the oleosin-chymosin fusion gene. Indicated in bold (nt 1625-1631) is the *NcoI* site containing the methionine start codon of the prochymosin sequence. The preceding spacer sequence (nt 1608-1630), replacing the oleosin stopcodon is underlined.  
20

Figure 7 shows a schematic drawing of plasmid M1830. The plasmid was constructed by replacing the Ura3 gene from pVT102-U (Gene 52: 225-233, 1987) with the Leu2 gene.  
25

Figure 8 shows a schematic drawing of plasmid M1830oleoGUS. A *BamHI*-GUS-*hindIII* fragment was inserted into the multiple cloning site of  
30 M1830, resulting in M1830GUS. A *B. napus* oleosin cDNA was furnished with *BamHI* sites at the 5' and 3' ends of the gene and inserted in frame and in the

right orientation in the *Bam*HI site of M1830GUS yielding plasmid M1830oleoGUS.

Figure 9. Sequence alignment of the isolated caleosin (SEQ ID NO.34) (this application) with the coding sequence of the reported caleosin gene (accession number AF067857) (SEQ ID NO.35). Indicated are the primers GVR979 and GVR980 used for the polymerase Chain Reaction and the one nucleotide change (position 69).

Figure 10. Nucleotide sequence of insert of pSBS2098 containing the phaseolin promoter- $\beta$  Glucuronidase (GUS)-phaseolin terminator sequence (SEQ ID NO.36). The GUS sequence and its deduced amino acid sequence (SEQ ID NO.37) is indicated in uppercase. The phaseolin promoter corresponds to nucleotide 1-1547, and the phaseolin terminator corresponds to nucleotide sequence 3426-4646. The terminator was furnished with a a KpnI site (nt 4647-4652) to facilitate cloning.

Figure 11. Nucleotide sequence of the phaseolin promoter-oleosinGUS-phaseolin terminator sequence (SEQ ID NO.38). The oleosinGUS coding sequence and its deduced aminoacid sequence is indicated (SEQ ID NOs.39 and 40). The phaseolin promoter corresponds to nucleotide 6-1554. The sequence encoding oleosin corresponds to nt 1555-2313, the intron in this sequence (nt 1908-2147) is indicated in italics. The GUS sequence corresponds to nt 2314-4191. The phaseolin terminator corresponds to nucleotide sequence 4192-5412.

Figure 12. Nucleotide sequence of the phaseolin promoter-caleosinGUS-phaseolin terminator sequence (SEQ ID NO.41). The caleosinGUS coding sequence and its deduced aminoacid sequence is indicated (SEQ ID NO.42). The phaseolin promoter corresponds to nucleotide 1-1545. The sequence encoding caleosin corresponds to nt 1548-2282, the NcoI restriction, which was used for the in-frame cloning and which separates the caleosin and GUS sequence (nt 2284-2289) is underlined. The GUS sequence corresponds to nt 2286-4163. The phaseolin terminator corresponds to nucleotide sequence 4164-5384.

Figure 13. Histochemical staining of  $\beta$ -Glucuronidase (GUS) activity in flax embryos bombarded with vectors pBluescriptIIKS+ (negative control), pSBS2098 (GUS) pSBS2037 (oleosinGUS), and pSBS2601 (caleosinGUS).

#### **DETAILED DESCRIPTION OF THE INVENTION**

5 In accordance with the subject invention, methods and compositions are provided for a novel means of production of heterologous proteins and peptides that can be easily separated from host cell components. In accordance with further embodiments of the invention methods and compositions are provided for novel uses of recombinant proteins produced by  
10 said methods.

In accordance with one aspect of the subject invention, methods and compositions are provided for a novel means of production of heterologous proteins and peptides in host cells that are easily separated from other host cell components. Purification of the protein, if required, is greatly simplified. The  
15 nucleic acid encoding the heterologous peptide may be part or all of a naturally occurring gene from any source, it may be a synthetic nucleic acid sequence or it may be a combination of naturally occurring and synthetic sequences. The subject method includes the steps of preparing an expression cassette comprising a first nucleic acid sequence capable of regulating the transcription of  
20 a second nucleic acid sequence encoding a sufficient portion of an oil body protein gene to provide targeting to an oil body and fused to this second nucleic acid sequence a third nucleic acid sequence encoding the polypeptide of interest; delivery and incorporation of the expression cassette into a host cell; production of a transformed organism or cell population in which the chimeric gene  
25 product is expressed and recovery of a chimeric gene protein product through specific association with an oil body. The heterologous peptide is generally a foreign polypeptide normally not expressed in the host cell or found in association with the oil-body.

The term "oil body protein" as used herein means a protein that  
30 can naturally associate with oil bodies or can be isolated using a standard oil body preparation protocol. An oil body preparation protocol is described in van Roijen and Moloney, 1995, Bio/Technology, 13:72-77. The oil body protein may

share sequence homology with other oil body proteins which may be oleosins or caleosins known in the art.

In one embodiment, the oil body protein is a plant oleosin and shares sequence homology with other plant oleosins such as the oleosin isolated from *Arabidopsis thaliana* (Figure 2 and SEQ.ID.NO:2) or *Brassica napus* (Figure 4 and SEQ.ID.NO:5). In another embodiment, the oil body protein is a plant caleosin and shares sequence homology with other plant caleosins such as the caleosin isolated from *Arabidopsis thaliana* shown in Figure 9 (SEQ.ID.NOs.34 and 35)

10 The term "heterologous polypeptide" as used herein means a polypeptide, peptide or protein that is not normally linked or fused to an oil body protein and is not normally expressed in association with oil bodies.

The term "nucleic acid sequence" refers to a sequence of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The term also includes modified or substituted sequences comprising non-naturally occurring monomers or portions thereof, which function similarly. The nucleic acid sequences of the present invention may be ribonucleic (RNA) or deoxyribonucleic acids (DNA) and may contain naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The sequences may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl, and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-amino adenine, 8-thiol adenine, 8-thio-alkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo 20 guanines, 8-amino guanine, 8-thiol guanine, 8-thioalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

30 The host cell may be selected from a wide range of host cells including plants, bacteria, yeasts, insects and mammals. In one embodiment the host cell is a plant cell. The use of plants to produce proteins of interest allows exploitation of the ability of plants to capture energy and limited nutrient input

to make proteins. The scale and yield of material afforded by production in plants allows adaptation of the technology for use in the production of a variety of polypeptides of commercial interest. The plant may be selected from various plant families including *Brassicaceae*, *Compositae*, *Euphorbiaceae*, *Leguminosae*,  
5 *Linaceae*, *Malvaceae*, *Umbelliferae* and *Graminae*.

In another embodiment the host cell is a bacterial cell. Bacterial host cells suitable for carrying out the present invention include *E. coli*, *B. subtilis*, *Salmonella typhimurium* and *Staphylococcus*, as well as many other bacterial species well known to one of ordinary skill in the art. Representative examples  
10 of bacterial host cells include JM109 ATCC No. 53323 and DH5 (Stratagene, LaJolla, California). Suitable bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the LacZ, the  $\beta$ -lactamase (penicillinase) and lactose promoter system (see  
15 Chang et al., Nature 275:615, 1978), the trp promoter (Nichols and Yanofsky, Meth in Enzymology 101:155, 1983) and the tac promoter (Russell et al., Gene 20:231, 1982).

In another embodiment, the host cell is a yeast cell. Yeast and fungi host cells suitable for carrying out the present invention include, among  
20 others *Saccharomyces cerevisiae*, the genera *Pichia* or *Kluyveromyces* and various species of the genus *Aspergillus*. Suitable expression vectors for yeast and fungi include, among others, YC<sub>p</sub>50 (ATCC No. 37419) for yeast, and the amdS cloning vector pV3 (Turnbull, Bio/Technology 7:169, 1989). Protocols for the transformation of yeast are also well known to those of ordinary skill in the art.  
25 For example, transformation may be readily accomplished either by preparation of spheroplasts of yeast with DNA (see Hinnen et al., PNAS USA 75:1929, 1978) or by treatment with alkaline salts such as LiCl (see Itoh et al., J. Bacteriology 153:163, 1983). Transformation of fungi may also be carried out using polyethylene glycol as described by Cullen et al. (Bio/Technology 5:369, 1987).

30 The host cell may also be a mammalian cell. Mammalian cells suitable for carrying out the present invention include, among others: COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g., ATCC No. CRL 6281), CHO (ATCC

No. CCL 61), HeLa (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573) and NS-1 cells. Suitable expression vectors for directing expression in mammalian cells generally include a promoter, as well as other transcriptional and translational control sequences. Suitable promoters include PMSG, pSVL, SV40, pCH 110, 5 MMTV, metallothionein-1, adenovirus Ela, CMV, immediate early, immunoglobulin heavy chain promoter and enhancer, and RSV-LTR. Protocols for the transfection of mammalian cells are well known to those of ordinary skill in the art. Representative methods include calcium phosphate mediated electroporation, retroviral, and protoplast fusion-mediated transfection (see 10 Sambrook et al., Molecular Cloning a Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press, 1989).

The host cell may also be an insect cell. Insect cells suitable for carrying out the present invention include cells and cell lines from *Bombyx* or *Spodotera* species. Suitable expression vectors for directing expression in insect 15 cells include Baculoviruses such as the *Autographa californica* nuclear polyhedrosis virus (Miller et al. 1987, in *Genetic Engineering*, Vol. 8 ed. Setler, J.K. et al., Plenum Press, New York) and the *Bombyx mori* nuclear polyhedrosis virus (Maeda et al., 1985, Nature 315:592).

In one embodiment the host cell is a plant and the chimeric 20 product is expressed and translocated to the oil bodies of the seed.

The use of an oil body protein as a carrier or targeting means provides a simple mechanism to recover proteins. The chimeric protein associated with the oil body or reconstituted oil body fraction is separated away from the bulk of cellular components in a single step (such as centrifugation size 25 exclusion or floatation); the protein is also protected from degradation during extraction as the separation also reduces contact of the proteins with non-specific proteases.

The invention contemplates the use of heterologous proteins, specifically enzymes, fused to oleosins and associated with oil bodies, or 30 reconstituted oil bodies for conversion of substrates in aqueous solutions following mixing of oil body fractions and substrate solutions. Association of the

enzyme with the oil body allows subsequent recovery of the enzyme by simple means (centrifugation and floatation) and repeated use thereafter.

In accordance with further embodiments of the invention methods and compositions are provided for the release of heterologous proteins and peptides fused to oleosin proteins specifically associated with isolated oil body or reconstituted oil body fractions. The subject method includes the steps of preparing an expression cassette comprising a first nucleic acid sequence capable of regulating the transcription of a second nucleic acid sequence encoding a sufficient portion of an oil body protein gene such as oleosin to provide targeting to an oil body and fused to this second nucleic acid sequence via a linker nucleic acid sequence encoding a amino acid sequence cleavable by a specific protease or chemical treatment a third nucleic acid sequence encoding the polypeptide of interest; such that the protein of interest can be cleaved from the isolated oil body fraction by the action of said specific chemical or protease.

For embodiments of the invention wherein the cleavage of heterologous proteins fused to oleosins associated with seed oil bodies is contemplated in germinating seed the expression cassette containing the heterologous protein gene so described above is modified to contain an additional second recombinant nucleic acid molecule comprising a first nucleic acid sequence capable of regulating expression in plants, particularly in germinating seed, more specifically seed embryo or other seed tissue containing oil bodies and under the control of this regulatory sequence a nucleic acid sequence encoding a protease enzyme, specifically a particular protease enzyme capable of cleavage of the fusion protein associated with said oil bodies to release a heterologous protein or peptide from the oil body, and a transcriptional and translational termination region functional in plants. It is desirable that the second recombinant nucleic acid molecule be so constructed such that the first and second recombinant nucleic acid sequences are linked by a multiple cloning site to allow for the convenient substitution of any one of a variety of proteolytic enzymes that may be used to cleave fusion proteins associated with oil bodies.

It is obvious to a person skilled in the art of plant molecular biology, genetics or plant breeding that the equivalent to the above modification to the expression cassette to allow release of proteins and peptides of interest in germinating seeds can be accomplished by other similar means. For example it is possible that the first recombinant nucleic acid molecule and the second recombinant nucleic acid molecule described above may be contained within two independent expression cassettes introduced into the genome of a plant independently. Additionally it is possible to sexually cross a first recombinant plant containing the first recombinant nucleic acid molecule integrated into its genome with a second recombinant plant with the second recombinant nucleic acid integrated into its genome to produce seed comprising both the first and second nucleic acid molecules.

For embodiments of the invention wherein the heterologous protein is to be produced in and potentially recovered from plant seeds the expression cassette will generally include, in the 5'-3' direction of transcription, a first recombinant nucleic acid sequence comprising a transcriptional and translational regulatory region capable of expression in plants, particularly in developing seed, more specifically seed embryo or other seed tissue that has oil body or triglyceride storage such as pericarp or cuticle, and a second recombinant nucleic acid sequence encoding a fusion peptide or protein comprising a sufficient portion of an oil body specific protein to provide targeting to an oil body, a heterologous protein of interest, and a transcriptional and translational termination region functional in plants. One or more introns may also be present within the oil body specific protein coding sequence or within the coding sequence of the heterologous protein of interest. The fusion peptide or protein may also comprise a peptide sequence linking the oil body specific portion and the peptide or protein of interest that can be specifically cleaved by chemical or enzymatic means. It is desirable that the nucleic acid expression cassette is constructed in such a fashion that the first and second recombinant nucleic acid sequences are linked by a multiple cloning site to allow for the convenient substitution of alternative second recombinant nucleic acid sequences comprising the oil body targeting sequence and any one of a variety

of proteins or peptides of interest to be expressed and targeted to oil bodies in seeds.

According to one embodiment of the invention the expression cassette is introduced into a host cell in a form where the expression cassette is stably incorporated into the genome of the host cell. Accordingly it is apparent that one may also introduce the expression cassette as part of a recombinant nucleic acid sequence capable of replication and or expression in the host cell without the need to become integrated into the host chromosome. Examples of this are found in a variety of vectors such as viral or plasmid vectors capable of replication and expression of proteins in the host cell. One specific example are plasmids that carry an origin of replication that permit high copy number such as the pUC series of *E. coli* plasmids additionally said plasmids modified to contain an inducible promoter such as the *LacZ* promoter inducible by galactose or IPTG.

In an alternative embodiment of the invention nucleic acid is stably incorporated into the genome of the host cell by homologous recombination. Examples of gene targeting by homologous recombination have been described for various cell types including mammalian cells (Mansour et al., 1988, Nature, 336, 348-352) and plant cells (Miao and Lam, 1995, Plant Journal, 7: 359-365). Introduction into the host cell genome of the protein of interest may be accomplished by homologous recombination of the protein of interest in such a fashion that upon recombination an expression cassette is generated which will generally include, in the 5'-3' direction of transcription, a first nucleic acid sequence comprising a transcriptional and translational regulatory region capable of expression in the host cell, a second nucleic acid sequence encoding a fusion protein comprising a sufficient portion of an oil body protein to provide targeting to an oil body and a heterologous protein, and a transcriptional and translational termination region functional in plants.

For embodiments of the invention wherein the production and recovery of the heterologous protein is contemplated from non-plant cells the expression cassette so described above is modified to comprise a first recombinant nucleic acid sequence comprising a transcriptional and translational

regulatory sequence capable of expression in the intended host production cell or organism. Promoter regions highly active in cells of microorganisms, fungi, insects and animals are well described in the literature of any contemplated host species and may be commercially available or can be obtained by standard methods known to a person skilled in the art. It is apparent that one means to introduce the recombinant molecule to the host cell is through specific infectious entities such as viruses capable of infection of the host modified to contain the recombinant nucleic acid to be expressed.

In a further embodiment of the invention it is contemplated that proteins other than plant oleosins and proteins with homology to plant oleosins that may specifically associate with triglycerides, oils, lipids, fat bodies or any hydrophobic cellular inclusions in the host organism or with reconstituted plant oil bodies may be fused to a recombinant protein and used in the manner contemplated. A system functionally equivalent to plant oleosins and oil bodies has been described in bacteria (Pieper-Fürst et al., 1994, J. Bacteriol. 176:4328 - 4337). Other proteins from additional sources such as, but not limited to; fungi, insects or animals, with equivalent regulatory and targeting properties may be known or discovered by a person skilled in the art.

Of particular interest for transcriptional and translational regulation in plants of the first recombinant nucleic acid molecule is a regulatory sequence (promoter) from an oil body protein gene, preferably an oil body protein gene expressed in dicotyledonous oil seeds. The expression of these genes in dicotyledonous oilseeds was found to occur much earlier than had hitherto been believed as reported in the literature. Thus, the promoters and upstream elements of these genes are valuable for a variety of uses including the modification of metabolism during phases of embryogenesis which precede the accumulation of storage proteins. Alternatively said promoter may also comprise a promoter capable of expression constitutively throughout the plant or a promoter which has enhanced expression within tissues or organs associated with oil synthesis. Of more particular interest is a promoter that expresses an oil body protein to a high level. Many plant species are tetraploid or hexaploid and may contain numerous copies of functional oil body protein

genes. As it is preferable to obtain a gene that is controlled by a promoter that expresses at high levels when compared to other oil body protein genes within the same species it may be advantageous to choose a diploid species as a source of oil body protein genes. An example is the diploid cruciferous plant  
5 *Arabidopsis thaliana*, wherein only two or three oil body protein genes are detected by southern blot analysis whereas the seeds contain oil body proteins as a high percentage of total protein.

The degree of evolutionary relationship between the plant species chosen for isolation of a promoter and the plant species selected to carry out the  
10 invention may not be critical. The universality of most plant genes and promoter function within dicotyledonous species has been amply demonstrated in the literature. Additionally to a certain extent the conservation of function between monocot and dicot genes has also been shown. This is apparent to a person skilled in the art that the function of any given promoter in any chosen  
15 species may be tested prior to practising the invention by simple means such as transient expression of marker gene promoter fusions in isolated cells or intact tissues. The promoter region typically comprises minimally from 100 bp 5' to the translational start of the structural gene coding sequence, up to 2.5 kb 5' from the same translational start.

20 Examples of nucleic acid encoding sequences capable of providing targeting to an oil body protein are oleosins genes obtainable from *Arabidopsis thaliana* or *Brassica napus* which provide for expression of the protein of interest in seed (See Taylor et al., 1990, Planta 181:18-26). The necessary regions and amino-acid sequences needed to provide targeting to the oil body reside in the  
25 highly hydrophobic central region of oil body proteins. The amino acid sequence necessary to provide targeting to the oil body for *Arabidopsis thaliana* oleosins contain amino acids 46-117 shown in SEQ.ID.NO.2. Similarly, the amino acid sequence necessary to provide targeting to the oil body for *Brassica napus* oleosins contains amino acids 60-132 shown in SEQ.ID.NO.5. In a  
30 preferred embodiment, the amino acid sequence necessary for targeting additionally contains the N-terminus of the oleosin which includes amino acids

1-45 (SEQ.ID.NO.2) and 1-60 (SEQ.ID.NO.5) for *Arabidopsis* and *Brassica*, respectively.

To identify other oil body protein genes having the desired characteristics, where an oil body protein has been or is isolated, the protein  
5 may be partially sequenced, so that a probe may be designed for identifying mRNA. Such a probe is particularly valuable if it is designed to target the coding region of the central hydrophobic domain which is highly conserved among diverse species. In consequence, a DNA or RNA probe for this region may be particularly useful for identifying coding sequences of oil body proteins from  
10 other plant species. To further enhance the concentration of the mRNA, cDNA may be prepared and the cDNA subtracted with mRNA or cDNA from non-oil body producing cells. The residual cDNA may then be used for probing the genome for complementary sequences, using an appropriate library prepared from plant cells. Sequences which hybridize to the cDNA under stringent  
15 conditions may then be isolated.

In some instances, as described above, the use of an oil body protein gene probe (conserved region), may be employed directly for screening a genomic library and identifying sequences which hybridize to the probe. The isolation may also be performed by a standard immunological screening  
20 technique of a seed-specific cDNA expression library. Antibodies may be obtained readily for oil-body proteins using the purification procedure and antibody preparation protocol described by Taylor et al. (1990, *Planta*, 181:18-26). cDNA expression library screening using antibodies is performed essentially using the techniques of Huynh et al. (1985, in *DNA Cloning*, Vol. 1, a  
25 Practical Approach, ed. D.M. Glover, IRL Press, pp. 49-78). Confirmation of sequence is facilitated by the highly conserved central hydrophobic region (see Figure1). DNA sequencing by the method of Sanger et al. (1977, *Proc. Natl. Acad. Sci. USA*, 74:5463-5467) or Maxam and Gilbert (1980, *Meth. Enzymol.*, 65:497-560) may be performed on all putative clones and searches for homology  
30 performed. Homology of sequences encoding the central hydrophobic domain is typically 70%, both at the amino-acid and nucleotide level between diverse species. If an antibody is available, confirmation of sequence identity may also

be performed by hybrid-select and translation experiments from seed mRNA preparations as described by Sambrook et al. (1990, Molecular Cloning, 2nd Ed., Cold Spring Harbour Press, pp. 8-49 to 8-51).

cDNA clones made from seed can be screened using cDNA probes  
5 made from the conserved coding regions of any available oil body protein gene (e.g., Bowman-Vance and Huang, 1987, J. Biol. Chem., 262:11275-11279). Clones are selected which have more intense hybridization with seed DNAs as compared to seedling cDNAs. The screening is repeated to identify a particular  
10 cDNA associated with oil bodies of developing seeds using direct antibody screening or hybrid-select and translation. The mRNA complementary to the specific cDNA is absent in other tissues which are tested. The cDNA is then used for screening a genomic library and a fragment selected which hybridizes to the subject cDNA. Of particular interest for transcriptional and translational regulation in plants of said second recombinant nucleic acid molecule is a  
15 regulatory sequence (promoter) from a gene expressed during the germination of seeds and the early stages of growth of a seedling, specifically a gene showing high levels of expression during the stage of mobilization of stored seed reserves, more specifically the promoter sequence from the glyoxisomal enzymes iso-citrate lyase or malate synthase. Information concerning genomic  
20 clones of iso-citrate lyase and malate synthase from *Brassica napus* and *Arabidopsis* that have been isolated and described has been published (Comai et al., 1989, Plant Cell 1: 293-300) and can be used by a person skilled in the art, by the methods described above, to isolate a functional promoter fragment. Other enzymes involved in the metabolism of lipids or other seed reserves during  
25 germination may also serve as a source of equivalent regulatory regions.

In order to identify oil body proteins, other than oleosins or caleosins, oil body preparations such as described in the art for the plants canola (Van Rooijen and Moloney, 1995, Bio/Technology 13: 72-77) and peanut (Jacks et al., J.A.O.C.S., 1990, 67: 353-361) and such as described for oil body-like  
30 granules in the bacterial species *Rhodococcus ruber* (Pieper-Fürst et al., 1994, J. Bacteriol. 176: 4328-4337) may be performed. From such preparations, individual proteins may be readily identified upon electrophoresis on a SDS

polyacrylamide gel. Proteins may be extracted from the polyacrylamide gel following the protocol of Weber and Osborn (J. Biol. Chem., 1969, 244: 4406-4412) and polyclonal antibodies against oil body proteins may be obtained using the protocol described by Taylor (1990, Planta, 181: 18-26). In order to isolate  
5 the corresponding cDNA clone, a cDNA expression library may then be screened with the antibody using techniques familiar to a skilled artisan (see for example: Huynh et al., 1985, in DNA cloning, Vol. 1, a Practical Approach, ed. D.M. Glover, IRL Press, pp 49-78).

For production of recombinant protein oleosin fusions in  
10 heterologous systems such as animal, insect or microbial species, promoters would be chosen for maximal expression in said cells, tissues or organs to be used for recombinant protein production. The invention is contemplated for use in a variety of organisms which can be genetically altered to express foreign proteins including animals, especially those producing milk such as cattle and  
15 goats, invertebrates such as insects, specifically insects that can be reared on a large scale, more specifically those insects which can be infected by recombinant baculoviruses that have been engineered to express oleosin fusion proteins, fungal cells such as yeasts and bacterial cells. Promoter regions highly active in viruses, microorganisms, fungi, insects and animals are well described in the  
20 literature and may be commercially available or can be obtained by standard methods known to a person skilled in the art. It is preferred that all of the transcriptional and translational functional elements of the initiation control region are derived from or obtained from the same gene.

For those applications where expression of the recombinant  
25 protein is derived from extrachromosomal elements, one may chose a replicon capable of maintaining a high copy number to maximize expression. Alternatively or in addition to high copy number replicons, one may further modify the recombinant nucleic acid sequence to contain specific transcriptional or translation enhancement sequences to assure maximal expression of the  
30 foreign protein in host cells.

The level of transcription should be sufficient to provide an amount of RNA capable of resulting in a modified seed, cell, tissue, organ or

organism. The term "modified " is meant a detectably different phenotype of a seed, cell, tissue, organ or organism in comparison to the equivalent non-transformed material, for example one not having the expression cassette in question in its genome. It is noted that the RNA may also be an "antisense  
5 RNA" capable of altering a phenotype by inhibition of the expression of a particular gene.

Ligation of the nucleic acid sequence encoding the targeting sequence to the gene encoding the polypeptide of interest may take place in various ways including terminal fusions, internal fusions, and polymeric fusions.  
10 In all cases, the fusions are made to avoid disruption of the correct reading frame of the oil-body protein and to avoid inclusion of any translational stop signals in or near the junctions. The different types of terminal and internal fusions are shown in Figure 1 along with a representation of configurations *in vivo*.

15 In many of the cases described, the ligation of the gene encoding the peptide preferably would include a linker encoding a protease target motif. This would permit the release of the peptide once extracted as a fusion protein. Potential cleavage sites which could be employed are recognition motifs for thrombin (Leu-Val-Pro-Arg-Gly, SEQ. ID. NO.9) (Fujikawa et al., 1972,  
20 Biochemistry 11:4892-4899), of factor Xa (Phe-Glu-Gly-Arg-aa, SEQ. ID NO.10) (Nagai et al., 1985, Proc. Natl Acad. Sci. USA, 82:7252-7255) collagenase (Pro-Leu-Gly-Pro, SEQ. ID. NO.11) (Scholtissek and Grosse, 1988, Gene 62:55-64) or Tobacco Etch Virus (TEV) protease (Glu-Asn-Leu-Tyr-Phe-Gln-Gly SEQ. ID NO.12) (Dougherty et al., 1989, Virology, 172: 302). Additionally, for uses where  
25 the fusion protein contains a peptide hormone that is released upon ingestion, the protease recognition motifs may be chosen to reflect the specificity of gut proteases to simplify the release of the peptide.

For those uses where chemical cleavage of the polypeptide from the oil body protein fusion is to be employed, one may alter the amino acid  
30 sequence of the oil body protein to include or eliminate potential chemical cleavage sites. For example, one may eliminate the internal methionine residues in the *Arabidopsis* oleosin at positions 11 and 117 by site directed mutagenesis to

construct a gene that encodes a oleosin that lacks internal methionine residues. By making a N-terminal fusion with the modified oleosin via the N-terminal methionine residue already present in the *Arabidopsis* oleosin, one may cleave the polypeptide of interest by the use of cyanogen bromide providing there are  
5 no internal methionines in said polypeptide. Similar strategies for other chemical cleavage agents may be employed. It should be noted that a variety of strategies for cleavage may be employed including a combination of chemical modification and enzymatic cleavage.

By appropriate manipulations, such as restriction, chewing back or  
10 filling in overhangs to provide blunt ends, ligation of linkers, or the like, complementary ends of the fragments can be provided for joining and ligation. In carrying out the various steps, cloning is employed, so as to amplify the amount of nucleic acid and to allow for analyzing the nucleic acid to ensure that the operations have occurred in proper manner. A wide variety of cloning  
15 vectors are available, where the cloning vector includes a replication system functional in *E. coli* and a marker which allows for selection of the transformed cells. Illustrative vectors include pBR332, pUC series, M13mp series, pACYC184, etc for manipulation of the primary nucleic acid constructs. Thus, the sequence may be inserted into the vector at an appropriate restriction site(s), the resulting  
20 plasmid used to transform the *E. coli* host, the *E. coli* grown in an appropriate nutrient medium and the cells harvested and lysed and the plasmid recovered. Analysis may involve sequence analysis, restriction analysis, electrophoresis, or the like. After each manipulation the nucleic acid sequence to be used in the final construct may be restricted and joined to the next sequence, where each of the  
25 partial constructs may be cloned in the same or different plasmids.

The mode by which the oil body protein and the protein to be expressed are fused can be either a N-terminal, C-terminal or internal fusion. The choice is dependant upon the application. For example, C-terminal fusions can be made as follows: A genomic clone of an oil body protein gene preferably  
30 containing at least 100 bp 5' to the translational start is cloned into a plasmid vehicle capable of replication in a suitable bacterial host (e.g., pUC or pBR322 in *E. coli*). A restriction site is located in the region encoding the hydrophilic C-

terminal portion of gene. In a plant oil body protein of approximately 18 KDa, such as the *Arabidopsis* oleosin, this region stretches typically from codons 125 to the end of the clone. The ideal restriction site is unique, but this is not absolutely essential. If no convenient restriction site is located in this region, one  
5 may be introduced by sitedirected mutagenesis. The only major restriction on the introduction of this site is that it must be placed 5' to the translational stop signal of the OBP clone.

With this altered clone in place, a synthetic oligonucleotide adaptor may be produced which contains coding sequence for a protease recognition site  
10 such as Pro-Leu-Gly-Pro (SEQ. ID. NO. 11) or a multimer thereof. This is the recognition site for the protease collagenase. The adaptor would be synthesized in such a way as to provide a 4-base overhang at the 5' end compatible with the restriction site at the 3' end of the oil body protein clone, a 4-base overhang at the 3' end of the adaptor to facilitate ligation to the foreign peptide coding  
15 sequence and additional bases, if needed, to ensure no frame shifts in the transition between the oil body protein coding sequence, the protease recognition site and the foreign peptide coding sequence. The final ligation product will contain an almost complete oil body protein gene, coding sequence for collagenase recognition motif and the desired polypeptide coding region all  
20 in a single reading frame.

A similar approach is used for N-terminal fusions. The hydrophilic N-terminal end of oil-body proteins permits the fusion of peptides to the N-terminal while still assuring that the foreign peptide would be retained on the outer surface of the oil body. This configuration can be constructed from similar  
25 starting materials as used for C-terminal fusions, but requires the identification of a convenient restriction site close to the translational start of the oil body protein gene. A convenient site may be created in many plant oil body protein genes without any alteration in coding sequence by the introduction of a single base change just 5' to the start codon (ATG). In plant oil body proteins thus far  
30 studied, the second amino acid is alanine whose codon begins with a "G". A-C transition at that particular "G" yields a *Nco I* site. As an illustration of such a modification, the context of the sequences is shown below:

3' ..TC TCA ACA ATG GCA ... Carrot Oil Body Protein (SEQ. ID. NO.13)

3' ..CG GCA GCA ATG GCG ... Maize 18KDa Oil Body Protein (SEQ. ID. NO.14)

A single base change at the adenine prior to the 'ATG' would yield  
5 in both cases CCATGG which is an *Nco I* site. Thus, modification of this base  
using the site-directed mutagenesis will introduce a *Nco I* site which can be used  
directly for the insertion of a nucleic acid coding sequence assuming no other  
*Nco I* sites are present in the sequence. Alternatively other restriction sites may  
be used or introduced to obtain cassette vectors that provide a convenient  
10 means to introduce foreign nucleic acid.

The coding sequence for the foreign peptide may require  
preparation which will allow its ligation directly into the introduced restriction  
site. For example, introduction of a coding sequence into the *Nco I* site  
introduced into the oil body protein coding sequences described above may  
15 require the generation of compatible ends. This may typically require a single  
or two-base modification by site-directed mutagenesis to generate an *Nco I* site  
around the translational start of the foreign peptide. This peptide is then excised  
from its cloning vehicle using *Nco I* and a second enzyme which cuts close to the  
translational stop of the target. Again, using the methods described above, a  
20 second convenient site can be introduced by site-directed mutagenesis. It has  
been suggested by Qu and Huang (1990, supra) that the N-terminal methionine  
might be removed during processing of the plant oil body proteins protein *in vivo*  
and that the alanine immediately downstream of this might be acylated. To  
account for this possibility, it may be necessary to retain the Met-Ala sequence at  
25 the N-terminal end of the protein. This is easily accomplished using a variety of  
strategies which introduce a convenient restriction site into the coding sequence  
in or after the Ala codon.

The resultant constructs from these N-terminal fusions would  
contain an oil body protein promoter sequence, an in-frame fusion in the first  
30 few codons of the oil body protein gene of a high value peptide coding sequence  
with its own ATG as start signal if necessary and the remainder of the oil body  
protein gene and terminator.

A third type of fusion involves the placing of a high value peptide coding sequence internally to the coding sequence of the oil body protein. This type of fusion requires the same strategy as in N-terminal fusions, but may only be functional with modifications in regions of low conservation, as it is believed that regions of high conservation in these oil body proteins are essential for targeting of the mature protein. A primary difference in this kind of fusion is the necessity for flanking protease recognition sites for the release of the protein. This means that in place of the single protease recognition site thus far described, it is necessary to have the protein of interest flanked by one or more copies of the protease recognition site.

Various strategies are dependant on the particular use and nucleic acid sequence of the inserted coding region and would be apparent to those skilled in the art. The preferred method would be to use synthetic oligonucleotides as linkers to introduce the high value peptide coding sequence flanked by appropriate restriction sites or linkers. Orientation is checked by the use of an asymmetrically placed restriction site in the high-value peptide coding sequence.

The heterologous polypeptide of interest to be produced as an oleosin fusion by any of the specific methods described herein, may be any peptide or protein. For example, proteins that alter the amino acid content of seeds may be used. These include genes encoding proteins high in essential amino acids or amino acids that are limiting in diets, especially arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Storage proteins such as the high lysine 10 KDa zein from *Zea mays* or the 2S high methionine Brazil Nut storage protein may be used. Alternatively synthetic or modified storage proteins may be employed such as peptides encoding poly-lysine or poly-phenylalanine or fusions of one or more coding regions high in essential amino acids. Proteins may also encode useful additives for animal feeds. These proteins may be enzymes for modification of phytate content in meal such as phytase, more specifically phytase from novel sources and having novel activities. Proteins may also

encode hormones useful for boosting productivity such as growth hormones or bovine somatotropin. Proteins may also encode peptides useful for aquaculture.

Proteins may also be those used for various industrial processes. Examples of such proteins include chitinase, glucose isomerase, collagenase, 5 amylase, xylanase, cellulase, lipase, chymosin, renin or various proteases or protease inhibitors. One may also express proteins of interest to the cosmetic industry such as collagen, keratin or various other proteins for use in formulation of cosmetics. Proteins of use to the food industry may also be synthesized including sweetener proteins such as thaumatin, and other flavour 10 enhancing proteins. Proteins that have adhesive properties may also be used.

Of particular interest are those proteins or peptides that may have a therapeutic or diagnostic value. These proteins include antigens, such as viral coat proteins or microbial cell wall or toxin proteins or various other antigenic peptides, peptides of direct therapeutic value such as interleukin-1- $\beta$ , the 15 anticoagulant hirudin, blood clotting factors and bactericidal peptides, antibodies, specifically a single-chain antibody comprising a translational fusion of the VH or VL chains of an immunoglobulin. Human growth hormone may also be produced. The invention is not limited by the source or the use of the heterologous polypeptide.

20 The nucleic acid sequence encoding the heterologous polypeptide of interest may be synthetic, naturally derived, or a combination thereof. Dependent upon the nature or source of the nucleic acid encoding the polypeptide of interest, it may be desirable to synthesize the nucleic acid sequence with codons that represent the preference of the organism in which 25 expression takes place. For expression in plant species, one may employ plant preferred codons. The plant preferred codons may be determined from the codons of highest frequency in the proteins expressed in the largest amount in the particular plant species of interest as a host plant.

The termination region which is employed will be primarily one of 30 convenience, since in many cases termination regions appear to be relatively interchangeable. The termination region may be native to the transcriptional initiation region, may be native to the nucleic acid sequence encoding the

polypeptide of interest, or may be derived from another source. Convenient termination regions for plant cell expression are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. Termination signals for expression in other organisms are well known  
5 in the literature.

A variety of techniques are available for the introduction of nucleic acid into host cells. For example, the chimeric nucleic acid constructs may be introduced into host cells obtained from dicotyledonous plants, such as tobacco, and oleaginous species, such as *Brassica napus* using standard *Agrobacterium*  
10 vectors by a transformation protocol such as that described by Moloney et al., 1989, Plant Cell Rep., 8:238-242 or Hinchey et al., 1988, Bio/Technol., 6:915-922; or other techniques known to those skilled in the art. For example, the use of T-DNA for transformation of plant cells has received extensive study and is amply described in EPA Serial No. 120,516; Hoekema et al., 1985, Chapter V, In: The  
15 Binary Plant Vector System Offset-drukkerij Kanters B.V., Alblasterdam; Knauf, et al., 1983, Genetic Analysis of Host Range Expression by *Agrobacterium*, p. 245, In: Molecular Genetics of the Bacteria-Plant Interaction, Puhler, A. ed., Springer-Verlag, NY; and An et al., 1985, EMBO J., 4:277-284. Conveniently, explants may be cultivated with *A. tumefaciens* or *A. rhizogenes* to allow for transfer of the  
20 transcription construct to the plant cells. Following transformation using *Agrobacterium* the plant cells are dispersed in an appropriate medium for selection, subsequently callus, shoots and eventually plantlets are recovered. The *Agrobacterium* host will harbour a plasmid comprising the *vir* genes necessary for transfer of the T-DNA to the plant cells. For injection and electroporation,  
25 (see below) disarmed Ti-plasmids (lacking the tumour genes, particularly the T-DNA region) may be introduced into the plant cell.

The use of non-*Agrobacterium* techniques permits the use of the constructs described herein to obtain transformation and expression in a wide variety of monocotyledonous and dicotyledonous plants and other organisms.  
30 These techniques are especially useful for species that are intractable in an *Agrobacterium* transformation system. Other techniques for gene transfer include biolistics (Sanford, 1988, Trends in Biotech., 6:299-302), electroporation

(Fromm et al., 1985, Proc. Natl. Acad. Sci. USA, 82:5824-5828; Riggs and Bates, 1986, Proc. Natl. Acad. Sci. USA 83 5602-5606 or PEG-mediated DNA uptake (Potrykus et al., 1985, Mol. Gen. Genet., 199:169-177).

In a specific application, such as to *Brassica napus*, the host cells  
5 targeted to receive recombinant nucleic acid constructs typically will be derived from cotyledonary petioles as described by Moloney et al., 1989, Plant Cell Rep., 8:238-242). Other examples using commercial oil seeds include cotyledon transformation in soybean explants (Hinchee et al., 1988, Bio/technology, 6:915-922) and stem transformation of cotton (Umbeck et al., 1981, Bio/technology,  
10 5:263-266).

Following transformation, the cells, for example as leaf discs, are grown in selective medium. Once shoots begin to emerge, they are excised and placed onto rooting medium. After sufficient roots have formed, the plants are transferred to soil. Putative transformed plants are then tested for presence of a  
15 marker. Southern blotting is performed on genomic nucleic acid using an appropriate probe, for example an *A. thaliana* oleosin gene, to show that integration of the desired sequences into the host cell genome has occurred.

The expression cassette will normally be joined to a marker for selection in plant cells. Conveniently, the marker may be resistance to a  
20 herbicide, eg phosphinotricin or glyphosate, or more particularly an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, chloramphenicol, or the like. The particular marker employed will be one which will allow for selection of transformed cells compared with cells lacking the introduced recombinant nucleic acid.

25 The fusion peptide in the expression cassette constructed as described above, expresses at least preferentially in developing seeds. Accordingly, transformed plants grown in accordance with conventional ways, are allowed to set seed. See, for example, McCormick et al. (1986, Plant Cell Reports, 5:81-84). Northern blotting can be carried out using an appropriate  
30 gene probe with RNA isolated from tissue in which transcription is expected to occur such as a seed embryo. The size of the transcripts can then be compared with the predicted size for the fusion protein transcript.

Oil-body proteins are then isolated from the seed and analyses performed to determine that the fusion peptide has been expressed. Analyses can be for example by SDS-PAGE. The fusion peptide can be detected using an antibody to the oleosin portion of the fusion peptide. The size of the fusion peptide obtained can then be compared with predicted size of the fusion protein.

Two or more generations of transgenic plants may be grown and either crossed or selfed to allow identification of plants and strains with desired phenotypic characteristics including production of recombinant proteins. It may be desirable to ensure homozygosity of the plants, strains or lines producing recombinant proteins to assure continued inheritance of the recombinant trait. Methods of selecting homozygous plants are well known to those skilled in the art of plant breeding and include recurrent selfing and selection and anther and microspore culture. Homozygous plants may also be obtained by transformation of haploid cells or tissues followed by regeneration of haploid plantlets subsequently converted to diploid plants by any number of known means, (eg: treatment with colchicine or other microtubule disrupting agents).

The desired protein can be extracted from seed that is preferably homozygous for the introduced trait by a variety of techniques, including use of an aqueous, buffered extraction medium and a means of grinding, breaking, pulverizing or otherwise disrupting the cells of the seeds. The extracted seeds can then be separated (for example, by centrifugation or sedimentation of the brei) into three fractions: a sediment or insoluble pellet, an aqueous supernatant, and a buoyant layer comprising seed storage lipid and oil bodies. These oil bodies contain both native oil body proteins and chimeric oil body proteins, the latter containing the foreign peptide. The oil bodies are separated from the water-soluble proteins and re-suspended in aqueous buffer.

If a linker comprising a protease recognition motif has been included in the expression cassette, a protease specific for the recognition motif is added to the resuspension buffer. This releases the required peptide into the aqueous phase. A second centrifugation step will now re-float the processed oil bodies with their attached proteins and leave an aqueous solution of the released peptide or protein. The foreign protein may also be released from the

oil bodies by incubation of the oil body fraction with a different oil body fraction that contains the specific protease fused to oleosin. In this manner the protease cleavage enzyme is removed with the oil bodies that contained the fusion protein with the protease recognition site leaving a product uncontaminated by protease. The desired peptide may be precipitated, chemically modified or lyophilized according to its properties and desired applications

In certain applications the protein may be capable of undergoing self-release. For example, the proteolytic enzyme chymosin undergoes self-activation from a precursor to an active protease by exposure of the precursor to low pH conditions. Expression of the chymosin precursor/oil body fusion protein to conditions of low pH will activate the chymosin. If a chymosin recognition site is included between the oil body protein and the chymosin protein sequences, the activated chymosin can then cleave the fusion proteins. This is an example of self release that can be controlled by manipulation of the conditions required for enzyme activity. Additional examples may be dependant on the requirement for specific co-factors that can be added when self-cleavage is desired. These may include ions, specific chemical co-factors such as NADH or FADH, ATP or other energy sources, or peptides capable of activation of specific enzymes. In certain applications it may not be necessary to remove the fusion protein from the oil-body protein. Such an application would include cases where the fusion peptide includes an enzyme which is tolerant to N or C-terminal fusions and retains its activity; such enzymes could be used without further cleavage and purification. The enzyme/oil body protein fusion would be contacted with substrate. It is also possible to re-use said oil bodies to process additional substrate as a form of an immobilized enzyme. This specific method finds utility in the batch processing of various substances. The process is also useful for enzymatic detoxification of contaminated water or bodies of water where introduction of freely diffusible enzyme may be undesirable. Said process allows recovery of the enzyme with removal of the oil bodies. It is also possible, if desired, to purify the enzyme - oil body protein fusion protein using an immunoaffinity column comprising an immobilized high titre antibody against the oil body protein.

Other uses for the subject invention are as follows. Oil body proteins comprise a high percentage of total seed protein, thus it is possible to enrich the seed for certain desirable properties such as high-lysine, high methionine, and the like, simply by making the fusion protein rich in the amino-  
5 acid(s) of interest could find utility of particular interest is the modification of grains and cereals which are used either directly or indirectly as food sources for livestock, including cattle, poultry, and humans. It may be possible to include, as the fusion peptide, an enzyme which may assist in subsequent processing of the oil or meal in conventional oilseed crushing and extraction, for example  
10 inclusion of a thermostable lipid-modifying enzyme which would remain active at the elevated crushing temperatures used to process seed and thus add value to the extracted triglyceride or protein product. Other uses of the fusion protein include improvement of the agronomic health of the crop. For example, an insecticidal protein or a portion of an immunoglobulin specific for an agronomic  
15 pest such as a fungal cell wall or membrane, could be coupled to the oil body protein thus reducing attack of the seed by a particular plant pest.

It is possible that the polypeptide/protein will itself be valuable and could be extracted and, if desired, further purified. Alternatively the polypeptide/protein or even the mRNA itself may be used to confer a new  
20 biochemical phenotype upon the developing seed. New phenotypes could include such modifications as altered seed-protein or seed oil composition, enhanced production of pre-existing desirable products or properties and the reduction or even suppression of an undesirable gene product using antisense, ribozyme or co-suppression technologies (Izant and Weintraub, 1984, Cell 36:  
25 1007-1015, Hazelhoff and Gerlach, 1988, Nature 334:585-591, Napoli, et al., 1990, Plant Cell, 2:279-289). While one embodiment of the invention contemplates the use of the regulatory sequence in cruciferous plants, it is possible to use the promoter in a wide variety of plant species given the wide conservation of oleosin genes. For example, the promoter could be used in various other  
30 dicotyledonous species as well as monocotyledonous plant. A number of studies have shown the spatial and temporal regulation of dicot genes can be conserved when expressed in a monocotyledonous host. The tomato rbcS gene

(Kyoizuka et al, 1993, Plant Physiol. 102:991-1000) and the Pin2 gene of potato (Xu et al, 1993 Plant Physiol. 101:683-687) have been shown to function in a monocotyledonous host consistent with their expression pattern observed in the host from which they were derived. Studies have also indicated expression  
5 from some dicotyledonous promoters in monocotyledonous hosts can be enhanced by inclusion of an intron derived from a monocotyledonous gene in the coding region of the introduced gene (Xu et al, 1994, Plant Physiol. 106:459-467). Alternatively, given the wide conservation of oleosin genes, it is possible for the skilled artisan to readily isolate oleosin genes from a variety of host  
10 plants according to the methodology described within this specification.

It is expected that the desired proteins would be expressed in all embryonic tissue, although different cellular expression can be detected in different tissues of the embryonic axis and cotyledons. This invention has a variety of uses which include improving the intrinsic value of plant seeds by  
15 their accumulation of altered polypeptides or novel recombinant peptides or by the incorporation or elimination of a metabolic step. In its simplest embodiment, use of this invention may result in improved protein quality (for example, increased concentrations of essential or rare amino acids), improved lipid quality by a modification of fatty acid composition, or improved or  
20 elevated carbohydrate composition. The invention may also be used to control a seed phenotype such as seed coat color or even the development of seed. In some instances it may be advantageous to express a gene that arrests seed development at a particular stage, leading to the production of "seedless" fruit or seeds which contain large amounts of precursors of mature seed products.  
25 Extraction of these precursors may be simplified in this case.

Other uses include the inclusion of fusion proteins that contain antigens or vaccines against disease. This application may be particularly relevant to improvements in health care of fish or other wildlife that is not readily assessable by conventional means as the crushed seed can be converted  
30 directly into a convenient food source. Other uses include the addition of phytase to improve the nutritional properties of seed for monogastric animals through the release of phosphate from stored phytate, the addition of

chlorophyllase to reduce undesirable chlorophyll contamination of seed oils, especially canola oil and addition of enzymes to reduce anti-metabolites, pigments or toxins from seeds. Additionally the fusion protein may comprise, an insecticidal or fungicidal protein such as magainin or secropin or a portion of an immunoglobulin specific for an agronomic pest, such as a fungal cell wall or membrane, coupled to the oil body protein thus improving seed resistance to pre and post harvest spoilage.

Applications for the use of chimeric proteins associated with the oil body fraction include as above enzymes that are tolerant of N or C-terminal fusions and retain activity. Enzymes associated with oil body suspensions can be mixed with simple or complex solutions containing enzyme substrates. After conversion of substrates to products the enzyme oleosin fusion is readily recovered by centrifugation and floatation and can be reused an indefinite number of times.

#### Examples

The following examples are offered by way of illustration and not by limitation.

**Example 1: Isolation of Plant Oleosin Gene.** Oil body proteins can be isolated from a variety of sources. The isolation of an oil body protein gene termed oleosin from the plant species *Arabidopsis thaliana* is described herein. Similar methods may be used by a person skilled in the art to isolate oil body proteins from other sources. In this example, a *Brassica napus* oleosin gene (described by Murphy et al, 1991, Biochim Biophys Acta 1088:86-94) was used to screen a genomic library of *A. thaliana* (cv. Columbia) constructed in the Lambda cloning vector EMBL3A (Obtained from Stratagene Laboratories) using standard techniques. The screening resulted in the isolation of a EMBL 3A clone (referred to as clone 12.1) containing a 15 kb genomic fragment which contains a oleosin gene from *A. thaliana*. The oleosin gene coding region is contained within a 6.6 kb *Kpn I* restriction fragment of this 15 kb fragment. The 6.6kb *KpnI* restriction fragment was further mapped and a 1.8 kb *Nco I* / *Kpn I* fragment containing the oleosin gene including approximately 850 nucleotides of 5' sequence, the complete coding sequence and the 3' region was isolated. This 1.8 kb fragment

was end filled and subcloned in the *Sma* I site of RFM13mp19. The 1.8 kb insert was further digested with a number of standard restriction enzymes and subcloned in M13mp19 for sequencing. Standard cloning procedures were carried out according to Sambrook et al. (Molecular Cloning: A Laboratory Manual 2nd ed., 1989, Cold Spring Harbour Laboratory Press.) The nucleotide sequence was determined and the 1.8 kb sequence of the *A. thaliana* oleosin gene is presented in Figure2 and SEQ ID No.1. This particular DNA sequence codes for a 18 KDa *A. thaliana* oleosin gene. The coding region contains a single intron. This gene was used for the construction of recombinant protein expression vectors. The gene may also be used for screening of genomic libraries of other species.

**Example 2: Modification of a Native Oleosin for Expression of Heterologous Proteins.** The DNA fragment described in example 1 that contains the oleosin gene and regulatory elements was incorporated into an expression cassette for use with a variety of foreign/alternative genes. The following illustrates the modification made to the native *A. thaliana* oleosin gene, especially the promoter and coding region, in order to use this gene to illustrate the invention. It is contemplated that a variety of techniques can be used to obtain recombinant molecules, accordingly this example is offered by way of illustration and not limitation. The *A. thaliana* oleosin gene described in example 1 was cloned as a 1803 bp fragment flanked by *Nco* I and *Kpn* I sites in a vector called pPAW4. The plasmid pPAW4 is a cloning vehicle derived from the plasmid pPAW1 which is a Bluescript plasmid (Clontech Laboratories) containing a *Brassica napus* Acetolactate synthase (ALS) gene (Wiersma et al., 1989, Mol Gen Genet. 219:413-420). To construct pPAW4, the plasmid pPAW1 was digested with *Kpn* I. The digested DNA was subjected to agarose gel electrophoresis and the fragment that contained the Bluescript plasmid vector backbone and a 677 base pair portion of the *B. napus* ALS gene was isolated and religated. This plasmid contains the following unique restriction sites within the insert: *Pst* I, *Nco* I, *Hind* III and *Kpn* I. This plasmid was called pPAW4. The 1803 bp *Nco* I - *Kpn* I *Arabidopsis* oleosin gene fragment was cloned between the *Nco* I and *Kpn* I sites in pPAW4. The resultant plasmid contained in addition to the Bluescript plasmid

sequences, a 142 bp *Pst* I - *Nco* I fragment derived from the *B. napus* ALS gene and the entire 1803 bp *Arabidopsis* oleosin gene. The 142 bp *Pst* I - *Nco* I fragment is present only as a "stuffer" fragment as a result of the cloning approach and is not used in oleosin expression constructs.

5           The resultant plasmid was used to further modify the *Arabidopsis* oleosin gene. Site-directed mutagenesis was used to introduce nucleotide changes at positions -2, -1 and +4 in the DNA sequence shown in figure 2. The changes made were: A to T (nucleotide position -2); A to C (nucleotide position -1) and G to A (nucleotide position +4). These nucleotide changes create a 6  
10 nucleotide *Bsp* H1 restriction endonuclease site at nucleotide positions -2 to +4. The *Bsp* H1 site (T/CATGA) encompasses the ATG initiation codon and provides a recessed end compatible with *Nco* I. A second modification was made by digestion with the enzymes *Eco* RV and *Msc* I which released a 658 bp fragment containing most of the coding sequence of the native oleosin. This digestion left  
15 blunt ends at both the *Eco* RV and *Msc* I sites. The cut vector was recircularized in the presence of an oligonucleotide linker containing the following unique restriction sites: *Hind* III, *Bgl* II, *Sal* I, *Eco* RI and *Cla* I. The recircularized plasmid containing all the 5' regulatory sequences of the oleosin gene, a transcriptional start site and an initiation codon embedded in a *Bsp* H1 site. Thirty-one bases  
20 downstream of this is a short polylinker containing unique restriction sites. This plasmid was called pOleoP1. The restriction map of this construct is shown in figure3.

          Introduction of any DNA sequence into pOleoP1, this particular cassette requires that the foreign DNA sequence may have, or be modified to  
25 have, a *Bsp* H1 or *Nco* I site at the initial ATG position. This will assure conservation of the distance between the "cap" site and the initiator codon. Alternatively restriction site linkers may be added to facilitate insertion into the cassette. The same restriction site can be chosen for the site of insertion of the 3' end of the gene or linkers may be added to introduce appropriate sites. The  
30 complete chimeric construct is then excised using the appropriate restriction enzyme(s) and introduced into an appropriate plant transformation vector.

**Example 3: Using the *Arabidopsis* Oleosin Promoter For Controlling Expression in Heterologous Plant Species.** To demonstrate expression of the oleosin promoter and to determine the amount of 5' regulatory region required for expression in transgenic plants, a small number of DNA constructs were made that contain the 5' transcriptional initiation region of the *Arabidopsis* oleosin gene joined to the coding region for  $\beta$ -glucuronidase (GUS). These constructs were prepared using PCR. The constructs are designated according to the amount of the oleosin 5' region contained, for example, the 2500 construct has approximately 2500 base pairs of the oleosin 5' region. The constructs were introduced into *Brassica napus* and tobacco and the expression of the  $\beta$ -glucuronidase (GUS) gene was measured as described in detail below. The constructs were made using standard molecular biology techniques, including restriction enzyme digestion, ligation and polymerase chain reaction (PCR). As an illustration of the techniques employed, the construction of the 800 construct is described in detail.

In order to obtain a DNA fragment containing approximately 800 base pairs from the 5' transcriptional initiation region of the *Arabidopsis* oleosin gene in a configuration suitable for ligation to a GUS coding sequence, PCR was used. To perform the necessary PCR amplification, two oligonucleotide primers were synthesized (Milligen-Bioscience, Cyclone DNA synthesizer). The first primer, the 5' primer, was called GVR10 and had the following sequence (also shown in SEQ ID NO.15):

5'-CACTGCAGGAACTCTCTGGTAA-3' (GVR10)

The italicized bases correspond to nucleotide positions -833 to -817 in the sequence reported in Figure2. The *Pst* I site is underlined. The additional nucleotides 5' of this sequence in the primer are not identical to the oleosin gene, but were included in order to place a *Pst* I site at the 5' end of the amplification product.

The second primer, the 3' primer, is designated as ALP 1 and has the following sequence (also shown in SEQ ID NO.16):

5'-CTACCCGGGATCCTGTTTACTAGAGAGAATG-3' (ALP 1)

This primer contains the precise complement (shown in italics) to the sequence reported in Figure2 from base -13 to -30. In addition, it contains a further 13 bases at the 5' end added to provide two (overlapping) restriction sites, *Sma* 1 (recognition CCCGGG) and *Bam*H1 (recognition GGATCC), at the 3' end of the amplification product to facilitate cloning of the PCR fragment. Both the *Sma* 1 and *Bam* H1 sites are underlined, the *Bam* H1 site is delineated by a double underline.

These two primers were used in a PCR amplification reaction to produce DNA fragment containing the sequence between nucleotides -833 and -13 of the oleosin gene that now contains a *Pst* 1 site at the 5' end and *Sma* 1 and *Bam* H1 sites at the 3' end. The template was the oleosin genomic clone 12.1 described in example 1.

The amplification product was called OLEO p800 and was gel purified and digested with *Pst* 1. The digestion product was gel purified and end filled using DNA polymerase Klenow fragment then cut with *Sma* 1 to produce a blunt ended fragment. This fragment was cloned into the *Sma* 1 site of pUC19 to yield the plasmid pUC OLEOp800. This plasmid contained the insert oriented such that the end of the amplified fragment which contained the *Pst* 1 site is proximal to the unique *Hind* III site in the pUC19 cloning vector and the end of the amplified fragment that contains the *Sma* 1 and *Bam* H1 site is proximal to the unique *Eco* RI site in the pUC19. This subclone now contains approximately 800 base pairs of 5' regulatory region from the *Arabidopsis* oleosin gene.

The promoter region contained within the plasmid pUC OLEOp800 was fused to the reporter gene GUS. This was accomplished by substituting the oleosin promoter region for a heat shock promoter fused to a GUS gene in the plasmid HspGUS1559. HspGUS1559 is a plasmid used as a binary vector in *Agrobacterium*, derived from the vector pCGN 1559 (MacBride and Summerfeldt, 1990, Plant Molecular Biology, 14, 269-276) with an insert containing heat shock promoter (flanked by *Bam* H1 sites), the  $\beta$ -glucuronidase open reading frame and a nopaline synthase terminator (derived from pB1221, Jefferson RA in Cloning Vectors 1988, Eds. Pouwels P., Enger-Valk BE, Brammer WJ., Elsevier Science Pub BV, Amsterdam section VII, A11). The binary plasmid

HspGUS1559 was digested with *Bam*H1 which resulted in the release of the heat shock promoter and permitted the insertion of a *Bam*H1 fragment in its place. pUC OLEOp800 was then cut with *Bam* H1 to yield a promoter fragment flanked by *Bam* H1 sites. This fragment was cloned into the *Bam* H1 sites of the plasmid HspGUS1559 to yield the *Agrobacterium* binary transformation vector pOLEOp800GUS1559. The other constructs were prepared by the same PCR method described above using the appropriate primers for amplifying the 2500 fragment, the -1200 fragment, the -600 fragment or the -200 fragment. These plasmids was used to transform *Brassica napus* and tobacco. GUS expression assays (Jefferson R.A., 1987, Plant Mol. Biol. Rep. 5 387-405) were performed on the developing seeds and on non-reproductive plant parts as controls. The results in *Brassica napus* expressed as specific activity of GUS enzyme are shown in Table I. The results in tobacco are shown in Table II. GUS expression reported is an average obtained from approximately five seeds from each of approximately five different transgenic plants.

These results demonstrate that the oleosin fragment from -833 to -13 used in the 800 construct contains sufficient information to direct specific expression of a reporter gene in transgenic *Brassica napus* embryos as early as heart stage and that the *Arabidopsis* oleosin promoter is capable of directing transcription in plants other than *Arabidopsis*.

It should be noted that the specific expression demonstrated here does not depend on interactions with the native terminator of an oleosin gene 3' end. In this example, the 3' oleosin terminator was replaced by a terminator derived from the nopaline synthase gene of *Agrobacterium*. Thus, the sequence in the 800 construct is sufficient to achieve the desired expression profile independent of ancillary sequences.

**Example 4: Use of Oleosin Promoter and Coding Sequences to Direct Fusion Proteins to the Oil Body Fraction of Seeds.** In this example, we have prepared a transgenic plant which expresses, under the control of the oil body promoter, fusion proteins which associate with oilbodies. The enzymatic properties of the inserted coding sequences are preserved while fused to the oleosin. In this example we use the  $\beta$ -glucuronidase enzyme derived from the microorganism

*E. coli*. was fused to the oleosin coding region (referred to as a oleosin/GUS fusion) under the control of the *Arabidopsis* oleosin promoter. In order to create an in-frame GUS fusion with the *Arabidopsis* oleosin, two intermediate plasmids were constructed referred to as pOThromb and pGUSNOS.

5           The plasmid pOThromb comprises the oleosin 5' regulatory region, the oleosin coding sequence wherein the carboxy terminus of the protein has been modified by addition of a thrombin cleavage site. The plasmid pGUSNOS contains the GUS enzyme coding region followed by the nos terminator polyadenylation signal. These two plasmids were joined to make a  
10 fusion protein consisting of the oleosin protein fused to the GUS enzyme by way of a linker peptide that is recognized by the endoprotease thrombin.

          These plasmids were constructed using PCR and the specific primers shown below. For the construction of pOThromb, a linker oligonucleotide named GVR01 was synthesized having the DNA sequence  
15 (shown in SEQ ID NO.17) of:

```

                10          20          30          40
5'AATCCCATGG ATCCTCGTGG AACGAGAGTA GTGTGCTGGC
CACCACGAGT ACGGTCACGG TC 3'   (GVR01)
                50          60
```

20           This DNA sequence contains from nucleotides 27-62 sequences complementary to the 3' end of the *Arabidopsis* oleosin coding sequence, from nucleotides 12-26 sequences encoding amino acids that comprise the coding region for a thrombin cleavage site, LVPRGS, and from nucleotides 5-14, the sequence for the restriction sites *Bam* *HI* and *Nco* *I*. A second primer referred to  
25 as GVR10 was also synthesized and consisting of the following DNA sequence (also shown in SEQ ID NO.18):

```

                10          20
5'-CACTGCAGGAACTCTCTGGTAAGC-3' (GVR10)
```

30           This DNA sequence contains from nucleotides 5-24 sequences homologous to the oleosin 5' flanking sequence -834 and -814. These two primers were used to amplify the promoter region (0.8 kb) of the *Arabidopsis* oleosin gene contained in the clone 12.1 described in example 1. The resultant

fragment was endfilled and cloned in the *Sma I* site of pUC19. This plasmid was called pOThrom which contained the oleosin promoter region, the oleosin coding sequence followed by a cleavage site for the enzyme thrombin and restriction sites for the insertion of the  $\beta$ -glucuronidase (hereinafter GUS).

5 In order to create an in frame GUS fusion with the *Arabidopsis* oleosin coding region now contained in pOThrom, a GUS gene with the appropriate restriction site was constructed by the use of PCR. An oligonucleotide referred to as GVR20 was synthesized and containing the following DNA sequence (also shown in SEQ ID NO.19):

10                   10                   20  
5'-GAGGATCCATGGTACGTCCTGTAGAAACC-3' (GVR20)

This oligonucleotide contains from nucleotides 9-29, sequences complementary to the GUS gene and from nucleotides 3-12 the sequence for the restriction sites *Bam HI* and *Nco I* to facilitate cloning. In order to create these  
15 restriction sites the fourth nucleotide of the GUS sequence was changed from T to G changing the TTA codon (Leu) into GTA (Val). The second primer used was the universal sequencing primer comprising the DNA sequence (also shown in SEQ ID NO.20):

                  10  
20 5'-GTAAAACGACGGCCAGT-3' (Universal Sequencing Primer)

The GVR20 and the Universal Sequencing Primer were used to amplify the GUS-nopaline synthase terminator region from the plasmid pBI121 (Clontech Laboratories). This fragment was endfilled and cloned in the *Sma I* site of pUC19. This plasmid was called pGUSNOS.

25 The plasmid pOThromb was digested with *Pst I* and *Nco I*, pGUSNOS was digested with *Nco I* and *Xba I*. The inserts of both these plasmids were ligated simultaneously into pCGN1559 cut with *Xba I* and *Pst I* to generate plasmid pCJOBPGUS. The plasmid pCJOBPGUS contained in the following order, the *Arabidopsis* oleosin 5' regulatory region, the oleosin coding region, a  
30 short amino acid sequence at the carboxy end of the oleosin coding sequence comprising a thrombin protease recognition site, the coding region for the  $\beta$ -glucuronidase gene followed by the nos terminator polyadenylation signal. The

fusion protein coded for by this particular DNA construct is designated as an oleosin/GUS fusion protein.

This plasmid pCJOBPGUS was digested with *Pst* I and *Kpn* I cloned into the *Pst*I and *Kpn* I sites of pCGN1559 resulting in plasmid  
5 pCJOBPGUS which was used as a binary vector in *Agrobacterium* transformation experiments to produce transgenic *B. napus*. Seeds from transgenic *Brassica napus* were obtained and tested for GUS activity. The transformed seeds showed GUS activity specifically associated with the oil body fraction. The results of these experiments are shown in Table III. The data  
10 demonstrate specific fractionation of the GUS enzyme to the oil body fraction. This example illustrates the expression and targeting of a bacterial derived enzyme specifically to the oil body fraction of transgenic plants.

One skilled in the art would realize that various modifications can be made to the above method. For example, a constitutive promoter may be  
15 used to control the expression of a oleosin/GUS fusion protein. In particular, the 35S promoter may also be used to control the expression of the oleosin/GUS fusion described above by replacing the *Arabidopsis* oleosin promoter with the 35S promoter from CaMV (available from the vector pBI 221.1, Clontech Laboratories) in the vector pCJOBPGUS. The resultant vector can contain in the  
20 following order, the CaMV 35S promoter, the oleosin coding region, a short amino acid sequence at the carboxy end of the oleosin coding sequence comprising a thrombin protease recognition site, the coding region for the  $\beta$ -glucuronidase gene followed by the nos terminator polyadenylation signal. This plasmid can be inserted into Bin 19 and the resultant plasmid may be introduced  
25 into *Agrobacterium*. The resulting strain can be used to transform *B.napus*. GUS activity can be measured in the oil body fraction.

**Example 5: Cleavage of Oleosin-Fusion Proteins.** In example 4 it was demonstrated that the targeting information contained within the oleosin is sufficient to target the protein oleosin/GUS fusion to the oil body. The  
30 oleosin/GUS fusion protein contains an amino acid sequence (LVPRGS SEQ ID NO.21), which separates the oleosin from GUS. This sequence is recognized by the protease thrombin, which cleaves this peptide sequence after the arginine

(R) amino acid residue. The transgenic seeds containing these oleosin/GUS fusions, were used to demonstrate the general utility of such a method of cleavage of a foreign peptide from intact oil bodies containing oleosin/foreign peptide-fusions. The oil body fraction that contained the oleosin/GUS fusion was resuspended in thrombin cleavage buffer which consisted of 50 mM Tris (pH 8.0), 150 mM NaCl, 2.5 mM CaCl<sub>2</sub>, 2% Triton X-100 and 0.5 % sarcosyl. Thrombin enzyme was added and the sample was placed for 30 minutes each at 45° C, 50° C and 55° C. Following this incubation oil bodies were recovered and tested for GUS activity. GUS enzymatic activity was found in the aqueous phase following this cleavage and removal of the oil bodies. This is shown in table IV. Western blot analysis confirmed the cleavage of GUS enzyme from the oleosin/GUS fusion protein. This example illustrates the cleavage and recovery of a active enzyme from a oleosin/enzyme fusion following biosynthesis and recovery of the enzyme in the oil body fraction of transgenic seeds.

**Example 6: Use of Fusion Proteins as Reusable Immobilized Enzymes.** In this example, oleosin/GUS fusion proteins that were associated with oilbodies were used as immobilized enzymes for bioconversion of substrates. Advantage was taken of the fact that enzymatic properties are preserved while fused to the oleosin and the oleosin is very specifically and strongly associated with the oil bodies even when the oil bodies are extracted from seeds. In this example it is demonstrated that said fusion enzymes can be used repeatedly and recovered easily by their association with the oil bodies. In order to demonstrate the reusable and stable GUS activity of the transgenic seeds, transgenic oil bodies were isolated from mature dry seeds as follows. The *Brassica napus* transgenic seeds containing a oleosin/GUS fusion protein were ground in extraction buffer A which consists of 0.15 M Tricine-KOH pH 7.5, 10 mM KCl, 1 mM MgCl<sub>2</sub> and 1 mM EDTA, 4°C to which sucrose to a final concentration of 0.6M was added just before use. The ground seeds in extraction buffer were filtered through four layers of cheesecloth before centrifugation for 10 minutes at 5000 x g at 4°C. The oil bodies present as a surface layer were recovered and resuspended in buffer A containing 0.6M sucrose. This solution was overlaid with an equal volume of Buffer A containing 0.1M sucrose and centrifuged at 18,000 x g for 20

minutes. This procedure was repeated twice with the purified oil body fraction (which contained the oilbodies and oleosin/GUS fusion proteins) and was resuspended in buffer A containing 1mM p-nitrophenyl  $\beta$ -D-glucuronide, a substrate for the GUS enzyme. After incubation, the conversion of the colorless substrate to the yellow p-nitrophenol was used as an indication of GUS activity in the suspensions of transgenic oil bodies. This illustrated the activity of the enzyme is maintained while fused to the oleosin protein and the enzyme is accessible to substrate while attached to the oil bodies. The oil bodies were recovered as described above. No GUS enzyme remained in the aqueous phase after removal of the oil bodies. The oil bodies were then added to fresh substrate. When the oil bodies were allowed to react with fresh substrate, conversion of substrate was demonstrated. This process was repeated four times with no loss of GUS activity. In parallel quantitative experiments, the amount of methyl umbelliferyl glucuronide (MUG) converted to methyl umbelliferone was determined by fluorimetry, and the oil bodies were recovered by flotation centrifugation and added to a new test tube containing MUG. The remaining buffer was tested for residual GUS activity. This procedure was repeated several times. The GUS enzyme showed 100% activity after using four uses and remained stably associated with the oil body fraction. These results are shown in table V. These experiments illustrate the immobilization and recovery of the active enzyme following substrate conversion. The stability of the GUS activity in partially purified oil bodies was established by measuring the GUS activity of the oil body suspension several weeks in a row. The half-life of the GUS activity when the oil-bodies are stored in extraction buffer at 4°C is more than 3 weeks.

#### **EXPRESSION OF OLEOSIN FUSION PROTEINS**

**Example 7: Expression of an Oleosin/IL-1- $\beta$  as a Fusion Protein.** To further illustrate the utility of the invention, the human protein interleukin 1- $\beta$  (IL-1- $\beta$ ) was chosen for biosynthesis according the method. IL-1- $\beta$  consists of 9 amino acids (aa); Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys (Antoni et al., 1986, J. Immunol. 137:3201-3204 SEQ. ID. NO.22). The strategy for biosynthesis was to place this nine amino acid protein at the carboxy terminus of the native oleosin

protein. The strategy further employed the inclusion of a protease recognition site to permit the cleavage of the IL-1- $\beta$  from the oleosin protein while fused to the oil bodies. In order to accomplish this, a recognition site for the endoprotease Factor Xa was incorporated into the construct. The protease

5 Factor Xa can cleave a protein sequence which contains amino acid sequence ile-glu-gly-arg. Cleavage takes place after the arginine residue. Based on these sequences, an oligonucleotide was synthesized which contained 18 nucleotides of the 3' coding region of the *A. thaliana* oleosin (base position 742-759, coding for the last six amino acids of the native protein), an alanine residue (as a result

10 of replacing the TAA stop codon of the native oleosin with a GCT codon for alanine), the coding sequence for the Factor Xa cleavage (four codons for the amino acids ile-glu-gly-arg) followed by the coding sequence for IL-1- $\beta$ . The oligonucleotide further comprised a TAA stop coding after the carboxy terminus lysine residue of IL-1- $\beta$  and adjacent to this stop codon, a *Sal* 1 restriction site

15 was added. The IL-1- $\beta$  coding sequence was designed using optimal codon usage for the *B.napus* and *A. thaliana* oleosin. It is apparent to those skilled in the art that maximal expression is expected when the codon usage of the recombinant protein matches that of other genes expressed in the same plant or plant tissue. This oligonucleotide was inserted into the *Arabidopsis* oleosin gene.

20 The modified oleosin gene was cut with *Pst* 1 and *Sal* 1 and joined to the nos terminator to obtain the plasmid called pCGBPILT. This plasmid contains, in the following order, the *Arabidopsis* oleosin promoter, the oleosin coding sequence, including the intron, and the IL-1- $\beta$  coding region joined at the carboxy terminus of the oleosin protein through a Factor Xa protease

25 recognition site and the nos terminator polyadenylation signal. This construct was inserted into the binary plasmid Bin 19 (Bevan, M., 1984, Nucl. Acids Res. 12:8711-8721) and the resultant plasmid was introduced into *Agrobacterium*. The resulting strain was used to transform *B. napus* and tobacco plants.

The *Arabidopsis* oleosin/IL-1- $\beta$  fusion was stably integrated into

30 the genomes of tobacco and *B. napus*. Northern analysis of embryo RNA isolated from different transformed tobacco plants showed the accumulation of *Arabidopsis* oleosin/IL-1- $\beta$  mRNA.

Oil body proteins from transformed tobacco seeds were prepared, and western blotting was performed. An antibody raised against a 22 KDa oleosin of *B. napus*, was used to detect the *Arabidopsis* oleosin/IL-1- $\beta$  fusion in the tobacco seeds. This antibody recognizes all the major oleosins in *B. napus* and *A. thaliana*. In addition, this antibody recognizes the tobacco oleosins. In oleosins extracted from transformed tobacco seeds the antibody recognized a 20 KDa-protein, which represents oleosin/IL-1- $\beta$  fusion oleosin. This fusion protein was not present in the untransformed tobacco seed. These results demonstrate the accumulation of oleosin/IL-1- $\beta$  fusion in tobacco. Similar expression and accumulation is seen in *Brassica napus* transformed with the oleosin/IL-1- $\beta$  fusion gene. These results further exemplify the utility of the method for the expression of heterologous proteins in plants.

**Example 8: Expression of Oleosin/Hirudin Gene Fusion in *B. napus*.** As a further example of the invention, the protein hirudin, derived from the leech (a segmented worm) was synthesized and fused to oleosin. Hirudin is an anti-coagulant which is produced in the salivary glands of the leech *Hirudo medicinalis* (Dodt et al., 1984, FEBS Lett., 65:180-183). The protein is synthesized as a precursor protein (Harvey et al., 1986, Proc. Natl. Acad. Sci. USA 83: 1084-1088) and processed into a 65 amino acid mature protein. The hirudin gene was resynthesized to reflect the codon usage of *Brassica* and *Arabidopsis* oleosin genes and a gene fusion was made with the C-terminal end of the *Arabidopsis* oleosin gene. The gene sequences for oleosin and hirudin were separated by codons for an amino acid sequence encoding a Factor Xa endoprotease cleavage site. The resulting plasmid was called pCGBHIRT. This plasmid contains, in the following order, the promoter region of the *Arabidopsis* oleosin gene, the coding sequence of the oleosin protein including the intron, a factor Xa cleavage site and the resynthesized hirudin gene followed by the nos terminator polyadenylation signal. This construct was inserted into the binary plasmid Bin 19 and the resultant plasmid was introduced into *Agrobacterium*. The resulting strain was used to transform *B.napus* and tobacco.

The *Arabidopsis* oleosin/hirudin fusion (OBPHIR) was stably integrated into the genomes of *N. tabacum* and *B. napus* respectively. Northern

analysis of embryo RNA isolated from different OBPHIR transformed plants showed the accumulation OBPHIR mRNA in *B.napus* seeds. Monoclonal antibodies raised against hirudin confirmed the stable accumulation of the oleosin/hirudin fusion in the seeds of transformed plants. Transgenic seeds  
5 containing an oleosin/hirudin were assayed after a year of storage at room temperature. No degradation of the oleosin/hirudin protein could be observed demonstrating the stability of the huridin in intact seeds.

The huridin can be cleaved from the oleosin by the use of the Factor Xa cleavage site built into the fusion protein. Upon treatment of the  
10 oilbody fraction of transgenic *Brassica napus* seeds, active huridin was released. These results are shown in Table VI. This example illustrates the utility of the invention for the production of heterologous proteins with therapeutic value from non-plant sources.

**Example 9: Fusion of Foreign Proteins to the N-terminus of Oleosin** In this  
15 example, a foreign protein was joined to the oleosin coding region via fusion to the N-terminus of the oleosin. As an illustration of the method, the GUS enzyme was fused in-frame to the *Arabidopsis* oleosin coding region described in example 1. In order to accomplish this, four DNA components were ligated to yield a GUS-oleosin fusion under the control of the oleosin promoter. These  
20 were: The oleosin 5' regulatory region, the GUS coding region, the oleosin coding region, and the *nos ter* transcription termination region. These four DNA components were constructed as follows:

The first of these components comprised the oleosin promoter isolated by PCR using primers that introduced convenient restriction sites. The  
25 5' primer was called OleoPromK and comprised the sequence (also shown as SEQ. ID. NO.23):

5'-CGC GGT ACC ATGG CTA TAC CCA ACC TCG-3'  
                    <sup>Nco1</sup>  
                    Kpn1

30 This primer creates a convenient Kpn 1 site in the 5' region of the promoter. The 3' primer comprised the sequence (also shown as SEQ. ID. NO.24):

5'-CGC ATCGATGTTCTTGTTTACTAGAGAG-3'

*Cla1*

This primer creates a convenient *Cla* 1 site at the end of the untranslated leader sequence of the oleosin transcribed sequence just prior to the ATG initiation codon in the native oleosin sequence. These two primers were used to amplify a modified promoter region from the native *Arabidopsis* oleosin gene. Following the reaction, the amplification product was digested with *Kpn* 1 and *Cla* 1 to yield a 870bp fragment containing the oleosin promoter and the 5' untranslated leader sequence. This promoter fragment is referred to as Kpn-OleoP-Cla and was ligated in the *Kpn* 2-*Cla* 1 sites of a standard subcloning vector referred to as pBS.

The second DNA component constructed was the GUS coding region modified to introduce the appropriate restriction sites and a Factor Xa cleavage site. In order to accomplish this, the GUS coding region in the vector PBI221 was used as a template in a PCR reaction using the following primers. The 5' primer was called 5'-GUS-Cla which comprised the following sequence (also shown as SEQ. ID. NO.25):

20 5'- GCC ATCGAT Nde 1 CAT ATG TTA CGT CCT GTA GAA ACC CCA-3'  
Cla 1

The 3' primer was referred to as 3'-GUS-FX-Bam and comprised the following nucleotide sequence (also shown as SEQ. ID. NO.26):

25 5' CGC GGATCC TCT TCC TTC GAT TTG TTT GCC TCC CTG C-3'  
*Bam* H1 Factor Xa  
 encoding DNA sequence  
 shown in boldface

This second oligonucleotide also encodes four amino acids specifying the amino acid sequence I-E-G-R, the recognition site for the endoprotease activity of factor Xa. The amplification product of approximately 1.8 kb comprises a GUS coding region flanked by a *Cla* I site at the 5' end and in place of the GUS termination codon, a short nucleotide sequence encoding the

four amino acids that comprise the Factor Xa endoprotease activity cleavage site. Following these amino acid codons is a restriction site for *Bam*H1.

The isolation of the oleosin coding region was also performed using PCR. To isolate this third DNA component, the *Arabidopsis* oleosin genomic clone was used as a template in a reaction that contained the following two primers. The first of these primers is referred to as 5'-Bam-Oleo and has the following sequence (also shown as SEQ. ID. NO.27):

10 5' CGC GGATCC ATG GCG GAT ACA GCT AGA 3'  
*Bam* H1

The second primer is referred to as 3'-Oleo-Xba and has the following sequence (also shown as SEQ. ID. NO.28):

5' TGC TCT AGA CGA TGA CAT CAG TGG GGT AAC TTA AGT 3'  
*Xba* 1

15 PCR amplification of the genomic clone yielded an oleosin coding region flanked by a *Bam* H1 site at the 5' end and a *Xba* 1 site at the 3' end. This coding sequence was subcloned into the *Bam* Hi and *Xba* 1 site of the subcloning vector pBS.

20 The fourth DNA component comprised the nopaline synthetase transcriptional termination region (*nos ter*) isolated from the vector pBI 221 as a blunt-ended *Sst* 1-*Eco*RI fragment cloned into the blunt-ended *Hind* III site of pUC 19. This subclone has a *Xba* 1 site at the 5' end and a *Hind* III site at the 3' end.

25 As a first step to assemble these four DNA components, the oleosin coding region and *nos ter* were first jointed by ligation of the *Bam* H1-*Xba* 1 fragment of the oleosin coding region with the *Xba* 1-*Hind* III fragment of the *nos ter* into *Bam* H1-*Hind* III digested pUC 19. This construct yielded a subclone that comprised the oleosin coding region joined to the *nos ter*. As a second step in the assembly of the DNA components, the oleosin promoter region was then  
30 joined to the modified GUS coding region by ligation of the *Kpn* 1-*Cla* 1 oleosin

promoter fragment to the *Cla* 1-*Bam* H1 fragment of the GUS coding region modified to contain the Factor Xa recognition site and subcloning these ligated fragments into pUC 19 cut with *Kpn* 1 and *Bam* H1.

To assemble all four DNA components, the *Kpn* 1-*Bam* H1 oleosin promoter fused to the GUS coding region was ligated with the *Bam* H1-*Hind* III oleosin coding region-*nos* *ter* fragment in a tripartite ligation with *Kpn*1-*Hind* III digested *Agrobacterium* binary transformation vector PCGN1559. The resultant transformation vector was called pCGYGON1 and was mobilized into *Agrobacterium tumefaciens* EHA 101 and used to transform *B. napus*.  
5 Transformed plants were obtained, transferred to the greenhouses and allowed to set seed. Seeds were analyzed as described by Holbrook et al (1991, Plant Physiology 97:1051-1058) and oil bodies were obtained. Western blotting was used to demonstrate the insertion of the GUS oleosin fusion protein into the oil body membranes. In these experiments, more than 80% of the GUS oleosin  
10 fusion protein was associated with the oil body fraction. No degradation of the fusion protein was observed. This example illustrates the utility of the method for the expression and recovery of foreign proteins fused to the N-terminus of oleosin.  
15

**Example 10: Expression of an Oleosin/Chymosin Fusion Protein.** As a further  
20 example of the invention, the bovine aspartic protease, chymosin - which is also frequently referred to in the art as rennin - was expressed as an oleosin fusion. Also exemplified here is the cleavage of an oleosin fusion protein by chemical means.

A complementary DNA clone containing a gene of interest may be  
25 obtained by any standard technique. For the purpose of this experiment, reverse transcription PCR was used to obtain a full length pre-prochymosin cDNA clone. RNA isolated from calf abomasum was used as the source material for the PCR and primers were designed in accordance with the sequence

described by Harris et al. (1982, Nucl. Acids Res., 10: 2177-2187). Subsequently, prochymosin was furnished with an *NcoI* recognition sequence (CCATGG) in such a way that the initiating methionine codon was in frame with the prochymosin cDNA. The Met-prochymosin sequence was ligated in frame to the  
5 3' coding sequence of an *A. thaliana* oleosin genomic sequence oleosin in which the TAA stopcodon had been replaced by a short spacer sequence (encoding LVPRGS SEQ ID NO.29) and an *NcoI* site. The complete sequence of a *HindIII* fragment containing the oleosin-spacer-Met-prochymosin sequence is shown in Fig. 6 and SEQ. ID.NO 6. This *HindIII* fragment was joined to a nopaline  
10 synthase terminator and cloned into the binary vector pCGN1559 (McBride and Summerfelt, 1990, Plant Mol. Biol. 14: 269-276). The resulting plasmid was called pSBSOTPTNT and introduced in *A. tumefaciens*. The resulting bacterial strain was used to transform *B. napus* plants.

Oil bodies from transformed *B. napus* plants were prepared and  
15 resuspended in 100mM Tris-Cl, pH 8.0. In order to demonstrate chemical cleavage of chymosin from the oleosin-spacer-Met-prochymosin fusion, the pH of the oil body suspension was lowered into two steps to pH 5.5 and pH 3.0, respectively using HCl. Oil bodies were subjected to these acidic conditions for several hours prior to Western blotting. Western blotting was performed using  
20 polyclonal antibodies raised against bovine chymosin and using commercially available chymosin (Sigma) as a positive control. The oleosin-spacer-Met-prochymosin fusion protein (approximately 62 kDa) could only be detected in oil body protein extracts obtained from transgenic *B. napus* seeds incubated at pH 8.0 and pH 5.0. No mature chymosin (35 kDa) was detected in protein  
25 extracts incubated under these conditions. The mature chymosin polypeptide was detected as the predominant molecular species in oil body protein extracts incubated at pH 3.0. In addition, oil body protein extracts incubated at pH 3.0 were the only extracts exhibiting chymosin activity as measured by milk-clotting

assay. In protein extracts isolated from untransformed control plants no specific cross-reactivity with anti-chymosin antibodies was detected.

**Example 11: Expression of an Oleosin/Cystatin Fusion Protein.** As a further example of the present invention, the expression of a protein that is toxic to insects is illustrated. The cysteine protease inhibitor, cystatin (OC-I), from *Oryza sativa* was expressed in a germination-specific manner in *Brassica napus* cv. Westar. The strategy for biosynthesis was to place the coding sequence for the complete 11.5 kDa OC-I protein downstream of the isocitrate lysase (ICL) promoter, isolated from *Brassica napus* (Comai et al., 1989, Plant Cell 1: 293-300).  
The ICL promoter has been shown to be functional for several days directly after germination of the seeds. Thus, this will allow for the pulse release of cystatin only for several days after germination when seedlings are most susceptible to the feeding of insects such as the flea beetle (*Phyllotreta cruciferae*) or the red turnip beetle (*Entomoscelis americana*).

The 313 bp sequence, encoding OC-I, from the cDNA clone OC 9b (Chen et al., 1992, Prot. Expr. and Purif., 3: 41-49) was amplified by PCR, using 5' and 3' specific primers, designed to introduce *BspHI* and *BamHI* sites for cloning purposes. The resulting fragment was cloned into pITG7, a vector containing the *nos* terminator of transcription. OC-I-*nos* was amplified from this plasmid by PCR, using the 5' primer specific to the OC-I coding sequence and the Universal primer (Stratagene). The resulting OC-I-*nos* fragment was cloned into the *SmaI* site of pBS(KS), excised with *BspHI* and *KpnI* and introduced into pUC18-ICL (plasmid containing the ICL promoter) at the *NcoI* and *KpnI* sites. The entire ICL-OC-I-*nos* cassette was removed by digestion with *PstI*, cloned into the plant binary vector pCGN 1547 (McBride and Summerfelt, 1990, Plant Mol. Biol. 14: 269-276) and designated pCGN-ICLOC. This plasmid was introduced into *Agrobacterium tumefaciens* EHA101 and the resulting strain was used to transform *Brassica napus* cv. Westar, using the cut petiole

transformation method (Moloney et al., 1989, Plant Cell Reports 8: 238-242). Transformation resulted in the stable integration of the ICL-OC-I -nos construct into the genome of *Brassica napus*. Northern blot analysis of poly-A<sup>+</sup> mRNA isolated from seedlings showed the accumulation of OC-I mRNA transcripts  
5 between one (1) to four (4) days after germination.

Protein extracts from the cotyledons of transformed *Brassica napus* seedlings were prepared using standard techniques (Sambrook et al., 1989, Molecular Cloning: a laboratory manual 2nd ed, Cold Spring Harbor Laboratory Press) and Western blot analysis was performed in order to  
10 determine if OC-I protein was produced. A polyclonal antibody raised against the truncated 10 kDa recombinant form of OC-I (Chen et al., 1992) was produced and allowed the detection of the complete OC-I protein (11.5 kDa) in extracts prepared from transformed *Brassica napus* seedlings. The OC-I protein was not detected in ungerminated seeds or in untransformed seeds or seedlings.  
15 The expression of OC-I was also found to be tissue specific, with the protein being found in cotyledons and hypocotyls but absent from roots and the first true leaves.

In order to prove functionality of the OC-I protein produced in the *Brassica napus* seedlings, a proteinase inhibitor assay (Rymerson et al.,  
20 manuscript in preparation) was performed, using the proteinase papain. OC-I produced in the seedlings was shown to significantly inhibit the activity of papain. The experiments described here, indicate that OC-I protein, cystatin, is produced in a germination and tissue specific manner and acts as a functional proteinase inhibitor in this system.

25 **Example 12: Expression of an Oleosin/Xylanase Fusion Protein.** As a further example of the present invention, the production of an industrial enzyme, xylanase, is illustrated. A variety of industrial applications have been reported for xylanases (Jeffries et al., 1994, TAPPI 77: 173-179; Biely, 1985, Trends

Biotechnol. 3: 286-290), including the conversion of the pulp and paper industry waste product xylan to useful monosaccharides.

The *xynC* gene encoding a highly active xylanase from the rumen fungus *Neocallimastix patriciarum* (Selinger et al., 1995, Abstract, 23rd Biennial Conference on Rumen Function, Chicago, Illinois) was joined in-frame to oleosin via a fusion to the C-terminus of the *Arabidopsis* oleosin coding region described in example 1. The *xynC* gene consists of an N-terminal catalytic domain preceded by a signal peptide. The xylanase gene lacking the ATG startcodon and partial signal peptide coding sequence was first amplified by PCR using the following 2 primers (also shown in SEQ ID NO 30 and SEQ ID NO 31):

10                  20                  30

5'-ATCTCTAGAATTCAACTACTCTTGCTCAAAG-3'

and

15                      10                      20  
5'-GGGTTGCTCGAGATTTCATCAATTTAT-3'

The PCR product was digested with *EcoRI* and *XhoI* and cloned into the *E. coli* expression vector pGEX4T-3 (Pharmacia) and designated pGEXxyn. Following expression and purification of the xylanase-glutathion-S-transferase fusion protein according to the protocol provided by the manufacturer, polyclonal antibodies against xylanase were obtained from rabbits immunized with thrombin-cleaved, purified recombinant xylanase.

In order to obtain the 1608 bp fragment containing the oleosin promoter and oleosin coding region, the construct pCGYOBPGUSA (van Rooijen and Moloney, 1995, Plant Physiol. 109: 1353-1361) was digested with *Pst*I and *Bam*HI. The xylanase coding region was obtained by digestion of pGEXxyn with *Eco*RI and *Xho*I. The oleosin fragment and xylanase fragment were cloned into pBluescript (pBS), previously digested with *Eco*RI and *Xho*I, resulting in

pBSOleXyn. In order to isolate the nopaline synthase (NOS) terminator region containing *XbaI* and *XhoI* cloning sites, the *BamHI* - *HindIII* fragment from pCGYOBPGUSA containing the NOS terminator sequence was subcloned in pBS to yield the intermediate plasmid pBSNos. Digestion of pBSNos with *XbaI* and  
5 *XhoI* and digestion of pBSOleXyn with *PstI* and *XhoI* yielded fragments containing the NOS terminator and the oleosin-xylanase fusion respectively and were ligated into the binary vector pCGN1559 which was digested with *PstI* and *XhoI*. The resulting binary vector containing the recombinant oleosin-xylanase fusion was named pCGOleXyn. Following introduction of pCGOleXyn into *A.*  
10 *tumefaciens*, *B. napus* cv Westar plants were transformed using the method of Moloney et al. (1989, Plant Cell Rep. 8: 238-242).

Accumulation of oleosin-XynC fusion protein in oil-bodies of transgenic canola plants was assessed by Western analysis. Probing of total seed protein extracts and oil body protein extracts with anti-XynC antiserum  
15 revealed the presence of a predominant band of 70 kDa on Western blots in both extracts. The predicted molecular weight of the oleosin-XynC fusion protein (68.2 kDa) and hence is in good agreement with the observed band. The fusion protein was absent in extracts from untransformed plants.

In order to evaluate functional activity of the oleosin-xylanase  
20 fusion proteins, xylanase enzyme assays using remazol brilliant blue-xylan (RBB-xylan) as described by Biely et al. (1988, Methods Enzymol. 160: 536-542) were carried out using oilbody immobilized xylanase. Xylanase activity was found to be associated almost exclusively with the oil body fraction and kinetic parameters were comparable to those of microbially expressed xylanase.

25 **Example 13: Expression of an Oleosin/Carp Growth Hormone Fusion Protein.**  
As a further example of this invention, the production of carp growth hormone (cGH) as an oleosin fusion protein is described. A DNA fragment containing the cGH coding region lacking its 22 amino acid signal sequence was amplified from

a plasmid containing on an insert a common carp (*Cyprinus carpio*) growth hormone cDNA (Koren et al., 1989, Gene 67: 309-315) using the PCR in combination with two cGH-specific primers. The amplified cGH fragment was fused in the correct reading frame and 3' to the *A. thaliana* oleosin using  
5 pOThromb (van Rooijen, 1993, PhD Thesis, University of Calgary) as a parent plasmid and employing cloning strategies similar to those outlined in the present application in e.g. examples 9 to 11 and well known to a person skilled in the art. In pOThromb a thrombin cleavage site was engineered 3' to the oleosin coding sequence. The oleosin-cGH fusion was introduced into the binary vector  
10 pCGN1559 (McBride and Summerfelt, 1990, Plant Mol. Biol. 14: 269-276) and the resulting construct was used to transform *A. tumefaciens*. The *Agrobacterium* strain was employed to transform *B. napus* cv Westar seedlings.

Seeds from transgenic *B. napus* plants were analysed for cGH expression by Western blotting using monoclonal antibodies against cGH. The  
15 expected 40 kDa oleosin-cGH fusion protein was specifically detected in oil body protein extracts containing the oleosin-cGH fusion protein. A 22 kDa polypeptide corresponding with cGH could be released from oil bodies upon treatment with thrombin, while no cGH was detected in oil body protein extracts from untransformed control plants.

20 **Example 14: Expression of an Oleosin/Zein Fusion Protein.** In order to demonstrate the utility of the instant invention for the production of improved meal, a gene specifying high levels of methionine residues, was expressed as an oleosin fusion in *B. napus* seeds. For the purpose of this experiment the gene encoding the corn seed storage protein zein (Kirihaara et al., 1988, Gene 71: 359-  
25 370) was used. The zein gene was fused 3' of the oleosin coding sequence and introduced in the binary vector pCGN1559 (McBride and Summerfelt, 1990, Plant Mol. Biol. 14: 269-276) employing cloning strategies similar to those described in the present application in e.g. examples 9 to 11 and well known to

the skilled artisan. The resulting recombinant plasmid was introduced in *A. tumefaciens* and used to transform *B. napus* cotyledonary explants. Amino acid analyses of canola meal of plants transformed with the oleosin-zein fusion construct indicated a significant increase in the levels of methionine in the meal  
5 when compared to untransformed plants.

**Example 15: Construction of an Oleosin/Collagenase Protein Vector.** As a further example of the invention, a vector containing an oleosin-collagenase fusion was constructed.

A 2.2 kbp fragment containing the collagenase gene from *vibrio*  
10 *alginolyticus* was PCR amplified from genomic bacterial DNA using primers in accordance with the published sequence (Takeuchi et al., 1992, Biochem. Journal, 281: 703-708). The fragment 2.2 kbp was then subcloned into pUC19 yielding pZAP1. Subsequently, the collagenase gene was introduced into pNOS8 containing the NOS terminator. The collagenase gene was ligated to the oleosin  
15 promoter and coding sequence of pThromb (van Rooijen, 1993, PhD Thesis, University of Calgary) containing a thrombin cleavage site and introduced into the binary vector pCGN1559 (McBride and Summerfelt, 1990, Plant Mol. Biol. 14: 269-276).

The collagenase construct may be introduced in a transgenic plant  
20 containing a second oleosin gene fusion to, for example, a gene encoding the enzyme chitinase isolated from tobacco (Melchers et al., 1994, Plant Journal 5: 469-480) and containing a collagenase recognition sequence engineered between the oleosin sequence and the second fusion protein. Introduction of the two fusion genes may be accomplished by sexual crossing of two lines which each  
25 contain one of the fusion genes or by transformation of a plant containing the first construct the second construct.

#### EXPRESSION IN PLANT HOSTS

**Example 16: Expression of Oleosin/GUS Fusions in Various Plant Species.** It is a feature of the present invention that a wide variety of host cells may be employed. In order to illustrate the expression of oleosin fusions in a number of plant species, the expression of the *A. thaliana* oleosin fused to the reporter gene GUS was assessed in the embryos of nine different plant species, including the monocotyledonous plant species *Zea mays* (corn).

Plasmid pCGYOBPGUS containing the intact *A. thaliana* oleosin gene with a carboxyl terminal fused GUS gene (van Rooijen et al., 1995, Plant Physiol. 109: 1353-1361) was used to transform oilseed embryos of the following plant species: *Brassica napus* (canola), *Helianthus annuus* (sunflower), *Carthamus tinctorius* (safflower), *Glycine max* (soybean), *Ricinus communis* (castor bean), *Linum usitatissimum* (flax), *Gossypium hirsutum* (cotton), *Coriandrum sativum* (coriander) and *Zea mays* (corn). Transformation was accomplished by particle bombardment (Klein et al., 1987, Nature, 327: 70-73) and plasmid pGN, containing a promoterless GUS gene was used as a control. Histochemical GUS staining (Klein et al., 1988, Proc. Natl. Acad. Sci. 85: 8502-8505) of the embryos was used to assess GUS expression.

The embryos of the 9 species transformed with plasmid pCGTYOBPGUS containing the oleosin-GUS fusion gene all exhibited substantial GUS expression as judged by histochemical staining. In contrast, no appreciable levels of GUS activity was detected in embryos transformed with the promoterless GUS construct.

#### EXPRESSION IN PROKARYOTES

**Example 17: Isolation of a *B. napus* Oleosin cDNA** The *Arabidopsis* oleosin gene described in Example 1 contains an intron, and as such is not suitable for use in a prokaryotic expression system. In order to express oleosin fusions in a microorganism such as bacteria, a coding sequence devoid of introns must be used. To accomplish this, a *B.napus* cDNA library was made using standard

techniques and was used to isolate oleosin cDNAs. Four clones were obtained and were called pcDNA#7, pcDNA#8, pcDNA#10 and pcDNA#12. These cDNA clones were partly sequenced, and one clone pcDNA#8, was sequenced completely. All the clones showed high levels of identity to oleosins. 5 pcDNA#10 was identical to pcDNA#12, but different from pcDNA#8 and pcDNA#7. The deduced amino acid sequence of the insert of pcDNA#8 is very similar to the *Arabidopsis* oleosin and is shown in figure 4. This coding region of oleosin can be used to isolate other oleosin genes or for expression of oleosin fusions in prokaryotic systems. It also provides a convenient coding region for 10 fusion with various other promoters for heterologous expression of foreign proteins due to the ability of the protein (oleosin) to specifically interact with the oilbody fraction of plant extracts.

**Example 18: Expression of a Oleosin/GUS Fusion in the Heterologous Host *E. coli*.** In order to further illustrate the invention, an oleosin/GUS gene fusion 15 was expressed in *E. coli* strain JM109. The oleosin cDNA pcDNA#8 described in example 17 was digested with *Nco* I and ligated into the *Nco* I site of pKKGUS, an expression vector containing the LacZ promoter fused to GUS. The plasmid pKKGUS was constructed by adding the GUS coding region to the vector pKK233 (Pharmacia) to generate the plasmid pKKoleoGUS and the anti-sense 20 construct pKKoeloGUS. This construct is shown in Figure 5. These plasmids were introduced into *E. coli* strain JM109 and expression was induced by IPTG. The *E. coli* cells were prepared for GUS activity measurements. In bacterial cells containing the vector pKKGUS, strong induction of GUS activity is observed following addition of IPTG. In cells containing pKKoleoGUS similar strong 25 induction of GUS activity was seen following addition of IPTG. In cells containing pKKoeloGUS (GUS in the antisense orientation) no induction over background was observed following the addition of IPTG. These results suggest that the oleosin/GUS fusion is active in bacteria. Although that activity

observed for the fusion product is less than the unfused product, the oleosin coding sequence was not optimized for expression in bacteria. It is apparent to those skilled in the art that simple modification of codons or other sequences such as ribosome binding sites could be employed to increase expression. The results are summarized in Table VII.

The fusion protein can be isolated from the bulk of the cellular material by utilizing the ability of the oleosin portion of the fusion proteins to specifically associate with oil bodies.

#### EXPRESSION IN FUNGI

Example 19: Expression of an Oleosin/GUS Fusion in the Heterologous *H st Saccharomyces cerevisiae*. As an example of the utility of the disclosed invention for expression in fungal systems, an oleosin-GUS fusion was expressed in *S. cerevisiae*. Plasmids pM1830OleoGUS, containing an oleosin-GUS fusion, and control plasmid pM1830 (Figs 7 and 8) were used to transform *S. cerevisiae* strain 1788 (Mata/Mat $\alpha$ ) an isogenic diploid of EG123 (MAT $\alpha$  *leu2-3,112 ura3-52trp1his4canI*; Kyung and Levin, 1992, Mol. Cel. Biol. 12: 172-182) according to the method of Elbe (1992, Biotechniques: 13: 18-19). Briefly, strain 1788 was grown on YPD (1% yeast extract, 2% peptone, 2% dextrose; Sherman et al., methods in yeast genetics, Cold Spring Harbor Laboratory Press) at 30° C for 1 day. The strain was then transformed with plasmids pM1830 and pM1830OleoGUS. Transformants were selected on synthetic media (SC, Sherman et al. Methods in yeast genetics, Cold Spring Harbor Laboratory Press), lacking leucine at 30° C for 3 days. Individual colonies were grown in SC (minus leucine) for 1 day, reinoculated into fresh medium at equal starting densities ( $OD_{600}$  = 0.05), then grown to mid-log phase ( $OD_{600}$  = 2.0-3.0). Cultures were centrifuged at 4,000 rpm for 5 min and the supernatant was removed. The pellet was resuspended in 100 mM Tris-Cl (pH 7.5), 1mM PMSF (phenyl methyl sulphonyl fluoride) and the cells were lysed using a French Press. GUS activity

measurements were done according to Jefferson (1987) and protein determination was done as described by Bradford et al. (1976, Anal. Biochem. 72: 248-254).

Western blotting using a polyclonal anti-oleosin antibody revealed the presence of a 90 kDa polypeptide, which is in agreement with the molecular weight deduced from the amino acid sequence of the fusion protein (89.7 kDa). No cross-reactivity was observed in extracts from the untransformed strain or in extract transformed with the control plasmid pM1830. Significant GUS activity could be detected in *S. cerevisiae* cells transformed with pM1830OleoGUS, while no appreciable levels of GUS activity were measured in untransformed cells or cells transformed with pM1830 (table VIII).

#### Example 20

##### Isolation of an *Arabidopsis* caleosin gene

An *Arabidopsis* silique cDNA library CD4-12 was obtained from the *Arabidopsis* Biological Resource Centre (ABRC, <http://aims.cps.msu.edu>) *Arabidopsis* stock centre and used as a template for the isolation of the caleosin gene from *Arabidopsis*. For the isolation of the caleosin gene the following primers were synthesized:

GVR979: 5' TACCATGGGGTCAAAGACGGAG 3'  
(SEQ ID NO.32)

The sequence of the caleosin identical to the 5' end of the *Arabidopsis* caleosin gene (as reported in the EMBL, Genbank and DDBJ Nucleotide Sequence Database under the accession number AF067857), is indicated in bold. Underlined is an NcoI restriction site to facilitate cloning.

GVR980: 5' ATCCATGGCGTAGTATGCTGTCTTGTCT 3'  
(SEQ ID NO.33)

The sequence complementary to the 3' end of the caleosin is indicated in bold. Underlined is an NcoI restriction site to facilitate cloning.

A Polymerase Chain Reaction (PCR) was carried out using GVR979 and GVR980 as primers and the cDNA library CD4-12 as a template. Note that this PCR has eliminated the stopcodon. This stopcodon is now

replaced by an NcoI restriction site which will allow for an in-frame fusion with the GUS sequence (See example 21). The resulted PCR fragment was isolated, cloned into pBluescript (Stratagene) and sequenced. The sequence of the PCR fragment and a comparison of this sequence with the published caleosin is shown in Figure 9. The isolated sequence encoding caleosin was identical to coding sequence of the reported caleosin gene accession number AF067857 except for one nucleotide at position 69 (See Figure 9). This nucleotide change did not result in an change in the deduced aminoacid sequence. The pBluescript vector containing the caleosin gene is called pSBS2099.

#### 10 Example 21

##### Construction of a plant expression vectors

*Construction of plant transformation vector pSBS2098.* An expression vector was constructed to allow for the seed specific expression of  $\beta$ -glucuronidase (GUS) in seeds. The  $\beta$ -glucuronidase gene was obtained from the plasmid pGUSN358S (Clontech laboratories). Standard molecular biology laboratory techniques (see eg: Sambrook *et al.* (1990) Molecular Cloning, 2<sup>nd</sup> ed. Cold Spring Harbor Press) were used to place the GUS gene between the phaseolin promoter and the phaseolin terminator derived from the common bean *Phaseolus vulgaris* (Slightom et al (1983) Proc. Natl Acad Sc USA 80: 1897-1901; Sengupta-Gopalan *et al.*, (1985) PNAS USA 82: 3320-3324. Standard molecular biology laboratory techniques (see eg: Sambrook *et al.* (1990) Molecular Cloning, 2<sup>nd</sup> ed. Cold Spring Harbor Press) were also used to furnish the phaseolin terminator with a KpnI site using the Polymerase Chain reaction (PCR) (see Figure 10). The phaseolin promoter contains a native EcoRI site at its 5'site. This EcoRI-phaseolin promoter - GUS - phaseolin terminator-KpnI sequence was cloned in the EcoRI - KpnI sites of cloned into pBluescript (Stratagene). This vector was called pSBS2098. A map of pSBS2098 is shown in Figure 13. The complete sequence of the insert of pSBS2098 is shown in Figure 10.

#### 30 *Construction of plant transformation vector pSBS2037*

An expression vector was constructed to allow for the seed specific expression of a oleosin- $\beta$ -glucuronidase (oleosinGUS) fusion in seeds. An *Arabidopsis*

oleosin gene as described Van Rooijen et al (1992) Plant Mol. Biol.18: 1177-1179) was placed upstream and in-frame (again the native stop codon was removed) to the GUS coding sequence of pSBS2099 using standard molecular biology laboratory techniques (see eg: Sambrook *et al.* (1990) Molecular Cloning, 2<sup>nd</sup> ed. Cold Spring Harbor Press). This vector was called pSBS2037. A map of pSBS2037 is shown in Figure 13 and the complete sequence of the insert of pSBS2037 is shown in Figure 11.

*Construction of plant transformation vector pSBS2601.* An expression vector was constructed to allow for the seed specific expression of a caleosin- $\beta$ -Glucuronidase (caleosinGUS) translational fusion in seeds.

The *Arabidopsis* caleosin gene as described in Example 20 was cloned as an NcoI fragment upstream (and in-frame) of the GUS coding. This vector was called pSBS2601. A map of pSBS2601 is shown in Figure 13 and the complete sequence of the insert of pSBS2601 is shown in Figure 12.

## 15 **EXAMPLE 22**

### **Transient expression of $\beta$ -glucuronidase, oleosin- $\beta$ -glucuronidase and caleosin- $\beta$ -glucuronidase in flax embryos**

Expression vectors pSBS2037, pSBS2098 and pSBS2601 were tested for their ability to transiently express GUS, oleosinGUS and caleosinGUS respectively. This transient expression was carried out bombarding flax embryos. As a control flax embryos were bombarded with gold particles with no DNA added. The subcellular location of the "free" GUS (as a result of transient expression of pSBS2098) the oleosinGUS fusion (as a result of expression of pSBS2037) and the caleosinGUS (as a result of expression of pSBS2601), and histochemical staining was determined/done essentially as described in Abenes et al (1997) Plant Cell reports 17: 1-7, with several minor modifications. In brief, the protocol was as follows: Three plates with 10 embryos each were separately bombarded with each plasmid (or just gold particles for the control). After particle bombardment one plate was used for histochemical staining (see Figure 13) and the embryos from the two remaining plates were combined for activity measurements. These were ground in 200  $\mu$ l oilbody extraction buffer (5 mM Tris-Cl pH 7.5, 0.4 M sucrose, 500 mM NaCl).

An aliquot was taken, this sample is referred to as "Total extract". The remainder is spun in an eppendorf microfuge at 15,000xg, resulting in a supernatant fraction of 150  $\mu$ l and an oilbody fraction. The oilbody fraction is resuspended in 150  $\mu$ l. 10 $\mu$ l of total extract, supernatant and oilbody fraction are added to 100  $\mu$ l GUS assay buffer (see Jefferson RA (1987) Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol Biol Rep 5: 387-405). This is done in duplicate. The reaction is incubated at 37°C for 2 hours after which 20 $\mu$ l of this reaction is added to 1ml 0.2 M Sodium Carbonate (Stop buffer). Fluorescence is measured according to Jefferson RA (1987). Plant Mol Biol Rep 5: 387-405. As can be seen in Figure 13, GUS expression is visible in panel B, C and D. This means that both oleosinGUS (panel C) and caleosinGUS (panel D) fusion proteins are expressed and result in a biologically active enzyme. Table IX shows the result of the GUS activity measurements. As can be seen from this table, both the "free" GUS, oleosinGUS and caleosinGUS are expressed. The "free" GUS is predominantly found in the supernatant fraction, whereas oleosinGUS and caleosinGUS are predominantly found in the oilbody fraction. This indicates that both oleosin and caleosin sequences are sufficient for the efficient targeting to oilbodies.

## ADDITIONAL APPLICATIONS OF THE INVENTION

The above examples describe various proteins that can be fused to an oil body protein and expressed in oil bodies in plants, bacteria and yeast. The above also provides the methodology to prepare such transgenic plants. Therefore one skilled in the art can readily modify the above in order to prepare fusion proteins containing any desired protein or polypeptide fused to an oil body protein in a variety of host systems. Several examples of other applications of the present invention are provided below.

- 10 a) **Construction of an Oleosin/Single Chain Antibody Fusion Protein.** As a further example of the invention, an antibody may be expressed in *B. napus*. Prior to the construction of an oleosin gene fusion, the deduced amino acid sequence of the coding region for the antibody may be back-translated using a *B. napus* codon usage table derived from several known *B.*  
15 *napus* genes and 'inside-out' recursive PCR (Prodomou & Pearl, 1992, Protein Eng. 5: 827-829) and yielding a synthetic scFv gene.

Gene fusion between the oleosin gene and the antibody gene can be accomplished by joining the synthetic antibody gene to the 5'end of the oleosin gene in a plasmid using cloning strategies well known to a person skilled  
20 in the art and similar to those outlined in the subject application in e.g. examples 9 to 11. The insert from the plasmid may be cloned into the binary vector pCGN1559 and used to transform *A. tumefaciens*. Cotyledonary petioles of *B. napus* may be transformed with the recombinant binary vector as described in Moloney et al. (1989, Plant Cell Reports, 8: 238-242).

- 25 Oil body extracts from transgenic *B. napus* plants may analysed by Western blotting using an anti oleosin antibody for the presence of the fusion protein.

b)                   **Combination of Two Oleosin Fusion Proteins to Release a Protein Product from Oil Bodies.** Two different oil body protein fusions associated with oil bodies can be used as a means to obtain a final product. For example, a transgenic *B. napus* may be obtained which contains a gene that  
5   comprises the GUS enzyme fused to the 3' coding sequence of oleosin separated by a collagenase protease recognition site. Oil bodies may be obtained from the seed of this plant. These oil bodies can be mixed with the oil bodies described above, which contains collagenase fused to oleosin. The collagenase activity of the oleosin/collagenase fusion protein oil bodies can release the GUS enzyme  
10   from the oleosin/GUS fusion proteins oil bodies. The GUS enzyme remains in the aqueous phase after removal of the oil bodies. No collagenase enzyme or contaminating oleosins will remain associated with the purified GUS enzyme illustrating the utility of the invention in obtaining easily purified proteins.

c)                   **Expression of a Oleosin/Phytase fusion protein in *B. napus*.** A  
15   microbial phytase from a *Aspergillus* may be isolated based on the published sequence (van Gorcom et al, European Patent Application 90202565.9, publication number 0 420 358 A1). This gene can be fused to the carboxy terminus of the oleosin protein using techniques described above and a collagenase recognition protease cleave site may be included to allow for  
20   separation of the phytase from the oil body if desired. The construct may contain, in the following order, the promoter region of the *Arabidopsis* oleosin gene, the coding sequence of the oleosin protein including the intron, a collagenase cleavage site and the phytase gene followed by the nos terminator polyadenylation signal. The construct can be inserted into the binary plasmid  
25   Bin 19 and the resultant plasmid introduced into *Agrobacterium*. The resulting strain can be used to transform *B.napus*. The seed of the transgenic plants will contain phytase activity. The phytase activity will be associated with the oil body fraction. The phytase activity is useful for the enhancement of meal for

monogastric animal feed. The phytase may be purified by treatment with collagenase as described in a), or the transgenic seed may be used as a feed additive.

d) **Expression of a Oleosin/Glucose isomerase.** The enzyme glucose isomerase can be expressed as a oleosin fusion protein by joining the coding sequence for the enzyme, (for example, described by Wilhelm Hollenberg, 1985, Nucl. Acid. Res. 13:5717-5722) to the oleosin protein as described above. The construct may be used to transform *B. napus*.

e) **Expression of a Oleosin/High Lysine Fusion Protein.** In order to increase the lysine content of transgenic seeds, a polylysine oligonucleotide may be added to the 3' coding region of the oleosin gene. For example, a repetitive oligonucleotide encoding a polylysine coding sequence can be made by synthesizing a (AAG)<sub>20</sub> oligonucleotide that is joined to the 3' coding region of the oleosin gene by replacement of the hirudin coding sequence contained within pCBOGHIRT plasmid described above in example 8 with the polylysine oligonucleotide through the use of cohesive restriction termini. The construct may contain, in the following order, the promoter region of the *Arabidopsis* oleosin gene, the coding sequence of the oleosin protein including the intron, 20 codons for the amino acid lysine followed by the nos terminator polyadenylation signal. The construct may be inserted into the binary plasmid Bin 19 and the resultant plasmid may be introduced into the *Agrobacterium*. The resulting strain can be used to transform *B. napus*.

f) **Expression of an Fungicidal Protein as an Oleosin Fusion Protein.** As a further example of the invention, a oleosin fusion protein may be constructed which encodes a protein that is toxic to fungi. For example, the gene for the enzyme chitinase isolated from tobacco (Melchers et al, 1994, Plant Journal 5:469-480) may be fused to the 3' coding region of oleosin under the control of the native oleosin promoter. Included in this construct may be an

oligonucleotide that encodes a collagenase recognition site located between the oleosin and chitinase coding regions. The expression of this construct will result in the production of a oleosin/chitinase fusion protein from which the chitinase enzyme can be released from the oleosin by treatment with collagenase. To this construct may be added a second chimeric gene capable of expression of a collagenase enzyme during seed germination. This second gene can comprise approximately 1.5 Kb of the 5' promoter region for isocitrate lyase, the collagenase coding sequence of *Vibrio alginolyticus* (Takeuchi et al., 1992, Biochemical Journal, 281:703-708) and the nos terminator. Isocitrate lyase is a glyoxysomal enzyme expressed under transcriptional control during early stages of seed germination (Comai et al., 1989, The Plant Cell, 1:293-300). This second construct therefore will express collagenase during the germination of the seed and mobilization of the oil body reserves. Expression of isocitrate lyase is restricted to germination and is not expressed in developing seeds. This second gene, joined to the oleosin/chitinase gene can be inserted into the binary vector Bin 19. The resultant vector may be introduced into *Agrobacterium* and used to transform *Brassica napus* plants. It is noted that the two genes may also be introduced independently or in two different plants which are then combined through sexual crossing. Seed from transgenic plants would be collected and tested for resistance to fungi.

**g) Expression of an Oleosin Fusion Protein that Provides Protection from Insect Predation.** As a further example of the invention, a fusion oleosin protein may be constructed which encodes a protein toxic to foraging insects. For example, the gene for cowpea trypsin inhibitor (Hilder et al., 1987, Nature, 330:160-163) may be used to replace the chitinase gene described in e). The expression of this construct will result in the production of a oleosin/trypsin inhibitor fusion protein from which the trypsin inhibitor can be released from the oleosin by treatment with collagenase. By replacement of the chitinase gene

in e) with the trypsin inhibitor, the construct also contains the collagenase gene under control of the germination specific promoter from the isocitrate lyase gene. This construct may be inserted into the binary vector Bin19. The resultant vector can be introduced into *Agrobacterium* and used to transform *Brassica*  
5 *napus* plants. Seed from transgenic plants were collected and tested for resistance to insect predation.

h) **Expression of an Enzyme to Alter Secondary Metabolites in Seeds.** In order to alter specific secondary metabolites in the seed, an enzyme encoding tryptophan decarboxylase (TDC) can be expressed in the seed as a  
10 fusion to oleosin. This particular enzyme (DeLuca et al., 1989, Proc. Natl. Acad. Sci. USA, 86:2582-2586), redirects tryptophan into tryptamine and causes a depletion of tryptophan derived glucosinolates. This lowers the amount of the antinutritional glucosinolates in the seed and provides a means to further reduce glucosinolate production in crucifer plant species. To accomplish this, a fusion  
15 protein may be constructed between the TDC gene and the oleosin coding region. The construct may contain, in the following order, the promoter region of the *Arabidopsis* oleosin gene, the coding sequence of the oleosin protein including the intron, the TDC gene followed by the nos terminator polyadenylation signal. The construct may be inserted into the binary plasmid  
20 Bin 19 and the resultant plasmid introduced into *Agrobacterium*. The resulting strain can be used to transform *B.napus*.

i) **Expression of Heterologous Proteins in Mammalian Cells.** The oil body protein - heterologous protein fusion may also be prepared in mammalian host cells. For example, an oleosin/GUS fusion may be inserted  
25 into a mammalian expression vector and introduced into mammalian cells as described below.

Expression of an oleosin/GUS fusion in mammalian cells would require the cloning of the GUS gene as described in example 17 in commercially

available mammalian expression vectors. For example, mammalian expression vectors pMSG, pSVL SV40, pCH 110, (all available from Pharmacia code No. 27-4506-01, 27-4509-01 and 27-4508-1 respectively) may be used. The oleosin/GUS fusion gene may be fused in the plasmid. These plasmids can be introduced into mammalian cells using established protocols (See eg. Introduction of DNA into mammalian cells (1995) Current Protocols in Molecular Biology, Ausubel et al. (ed) Supplement 29, Section 9). Accumulation of the oleosin/GUS transcript in mammalian cells can be determined after preparation of mammalian cell RNA (See eg. Direct analysis of RNA after transfection (1995) Current Protocols in Molecular Biology, Ausubel et al. (ed) Supplement 29, Section 9.8), northern blotting, and hybridization of this northern blot to a <sup>32</sup>P labelled *Brassica* oleosin cDNA as described in Example 18. After preparation of a total protein extract from the transfected mammalian cell culture, GUS activity can be measured, demonstrating the accumulation of the oleosin/GUS protein. Alternatively, immunoblotting can be performed on this protein extract using commercially available GUS antibodies and/or oleosin antibodies.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

**Table I. Expression of *Arabidopsis* oleosin chimeric promoter constructs in transgenic *Brassica napus*.**

Promoter Construct (GUS fusion)	Expression of GUS Activity (pmol/MU/mg protein/min)				
	Early Seed (torpedo)	Root	Leaf	Stem	Late Seed (cotyledon)
2500	7709	444	47	88	11607
1200	1795	-	-	-	8980
800	475	-	-	-	7130
600	144	-	-	-	1365
200	65	260	6	26	11
control	14	300	6	30	14

5

Oleosin promoter-GUS fusions were constructed as described in example 3. Included are GUS values obtained from a control non-transformed plant. A (-) indicated the tissue was not tested. Units are picomoles of methyl umbelliferone (product) per mg protein per minute.

**Table II. Expression of *Arabidopsis* oleosin chimeric promoter constructs in transgenic tobacco (*Nicotiana tabacum*).**

Promoter Constructs (GUS fusions)	GUS Activity in Seeds (pmol/MU/mg protein/min)
2500	11330
800	10970
Control	0

5

Oleosin promoter-GUS fusions were constructed as described in example 3. Included are GUS values obtained from a control non-transformed plant. Units are picomoles of methyl umbelliferone (product) per mg protein per minute.

**Table III. Specific partitioning of GUS/oleosin fusions into oil bodies when expressed in transgenic *Brassica napus* plants.**

Plant Number	Percent GUS Activity in Oil Bodies (%)	GUS Activity in Oil bodies	GUS Activity 100,000 X g Supernatant	GUS Activity in 100,000 X g Pellet
A1	88	493	1	67
B7	90	243	5	22
control	0	0	0	0

5

Plants were transformed with an oleosin/GUS fusion protein under the control of the *Arabidopsis* oleosin promoter. Transformed seeds were obtained and fractionated. The initial fractionation consisted of grinding the seeds in 1.5 mL of buffer A consisting of 15 mM Tricine-KOH, pH 7.5, 10 mM KCl, 1 mM Mg Cl<sub>2</sub>, 1  
10 mM EDTA, 100 mM sucrose followed by centrifugation at 14,000 X g for 15 minutes at 4°C. From this three fractions were obtained consisting of a floating oil body layer, an aqueous layer and a pellet. The oil body fraction was recovered and assayed for GUS activity. The remaining aqueous phase was further centrifuged for 2 hours at 100,000 X g. The pellet and supernatant from  
15 this centrifugation was also tested for GUS activity. Units are nmol MU per mg protein per min.

Table IV. Cleavage of GUS enzyme from oleosin/GUS fusions associated with oil bodies derived from transgenic *Brassica napus* containing an oleosin/GUS fusion protein.

5

GUS Activity (nmol product/mg protein/min)			
Fraction	Before Cleavage	After Cleavage	% Activity
Oil bodies	113	26.4	24
100,000 X g supernatant	14.3	83.6	76
100,000 X g pellet	15.7	-	-

Oil bodies containing an oleosin/GUS fusion protein were subjected to cleavage using the endopeptidase thrombin as described in example 5. Values shown are GUS activities before and after cleavage with thrombin. The values are also  
10 expressed as a percentage of total GUS activity released following enzyme fusion. Units are nmol methyl umbelliferone per mg protein/min.

**Table V. Reuse of oil body associated enzymatic activities.**

# Times Oil Bodies Washed	% GUS Activity	
	Oil bodies	Supernatant
1	100	8 ± 5
2	118 ± 7	5 ± 3
3	115 ± 8	3 ± 4
4	119 ± 8	1 ± 20

- 5 Oil bodies containing an oleosin/GUS protein were isolated from the seeds of transgenic *Brassica napus*. The oil bodies were added to the fluorometric GUS substrate MUG and allowed to react for one hour. The oil bodies were then recovered and added to a new tube containing the substrate and allowed to react for one hour again. This process was repeated a total of four times. The
- 10 table illustrates the reusable activity of the GUS enzyme while still associated with the oil bodies. Values are normalized to 100% as the GUS activity of original oil body isolates.

**Table VI. Recovery of active hirudin following synthesis of hirudin in plant seeds.**

Treatment	Thrombin Units Per Assay	Antithrombin Units per mg Oil Body Proteins
Buffer only	0.143	0
Wild-type seed	0.146	0
Wild-type seed + factor Xa	0.140	<0.001
Transformed (uncut)	0.140	<0.001
Transformed + factor Xa	0.0065	0.55

- 5 Oil bodies containing a hirudin/GUS fusion protein were isolated according to the method and treated with the endoprotease Factor Xa inhibition assay using N-p tosyl-gly-pro-arg-p-nitro anilide (Sigma). Hirudin activity was measured by the use of a thrombin in the method of Dodt et al (1984, FEBS Lett. 65, 180-183).
- 10 Hirudin activity is expressed as thrombin units per assay in presence of 255  $\mu$ g of oil body proteins, and also as antithrombin units per mg oil body protein.

**Table VII: Expression of active oleosin/GUS fusions in *E. coli*.**

Plasmid	Gus Activity
pKK233-2	2.5
pKKoeloGUS	3.1
pKKoleoGUS	28.1
pkkGUS	118.2

- 5 As described in example 22, oleosin/GUS fusions were expressed in *E. coli*. Cells were grown, induced with ITPG and GUS activity measured.

**Table VIII: GUS activity of total extracts of untransformed *S. Cerevisiae* strain 1788, transformed with M1830 and M1830oleoGUS**

<i>S. Cerevisiae</i> strain 1788	Specific Activity (pmol MU.min <sup>-1</sup> .μg prot <sup>-1</sup> )
untransformed	0.001
transformed with M1830	0.001
Transformed with M1830 OleosinGUS	41.3

Table IX. Transient GUS activity as a result of bombardment with plasmids pSBS2037, pSBS2098 and pSBS2601. The percentage of oilbody targeting was calculated as follows: (GUS activity on oilbodies) / (GUS activity on oilbodies + GUS activity in supernatant)) X 100%. As indicated in the text every experiment was done in duplicate.

	Plasmid used	protein produced	Activity nmol/l MU produced in cuvet	Activity minus control	% targeting to oil body
Total extract	no plasmid		72	-	
Total extract	no plasmid		55	-	
Total extract	2037	oleosinGUS	394	294.5	
Total extract	2037	oleosinGUS	457	357.5	
Total extract	2098	GUS	1561	1461.5	
Total extract	2098	GUS	1728	1628.5	
Total extract	2061	caleosinGUS	217	117.5	
Total extract	2061	caleosinGUS	170	70.5	
supernatant	no plasmid		58	-	
supernatant	no plasmid		56	-	
supernatant	2037	oleosinGUS	66	9.0	
supernatant	2037	oleosinGUS	65	8.0	
supernatant	2098	GUS	799	742.0	
supernatant	2098	GUS	1356	1299.0	
supernatant	2061	caleosinGUS	61	4.0	
supernatant	2061	caleosinGUS	64	7.0	
oilbody	no plasmid		50	-	
oilbody	no plasmid		46	-	
oilbody	2037	oleosinGUS	203	145	94
oilbody	2037	oleosinGUS	244	186	96
oilbody	2098	GUS	94	36	5
oilbody	2098	GUS	103	45	3
oilbody	2061	caleosinGUS	98	40	91
oilbody	2061	caleosinGUS	102	44	86